Detection of mutation and polymorphism in marker genes of cervical carcinoma in Bangladeshi patients

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CERTIFICATE

This is to certify that the thesis entitled **"Detection of mutation and polymorphism in marker genes of cervical carcinoma in Bangladeshi patients"** submitted by **Fatima Tuj Zohura**, Roll No. Curzon 02 of Ruqayyah Hall, Registration No. 221/2013-2014, carried out her research under our supervision. This is further to certify that it is an original work and suitable for partial fulfilment of the degree of Master of Philosophy (M.Phil.) in Genetic Engineering and Biotechnology, University of Dhaka.

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Dedicated to

My beloved parents

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ABSTRACT

Cervical cancer is a gynaecologic cancer type that develops in the cervix. Annually 528,000 new cases of cervical cancer are reported with 266,000 deaths, which account for 8% mortality of all female cancer patients. Infection with specific human papillomavirus (HPV) types is considered the most severe risk factor for cervical cancer. In the context of our socioeconomic conditions, an increasing burden of this disease and high mortality rate prevail in Bangladesh. Although several researches related to the epidemiology, HPV vaccination, and treatment modalities have been carried out in our country, any research on mutation locations and frequencies in cervical cancer in Bangladesh is yet to be done. Among the high number of genes involved in different signal transduction and cell growth regulation pathways, five different genomic regions within the top three most frequently mutated genes in COSMIC database with a key role in the development of cervical cancers were selected to study mutation frequency in our patients. These genes are *EGFR* (Epidermal Growth Factor Receptor), *KRAS* (Kirsten rat sarcoma), and *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha). DNA from 46 cervical tissue samples were extracted and amplified by PCR, using 1 set of primers designed for *EGFR* and 2 sets of primers designed for two different regions of both *PIK3CA* and *KRAS* gene. PCR products were purified and sequenced through Sanger cycle sequencing strategy. *In silico* analysis was done in two steps: nucleotide sequence analysis and its corresponding amino acid analysis. The mutated protein structures were determined by homology modelling using SWISS-MODEL. In total, 39 mutations were found in 28 patient samples. Eleven different mutations (23.91%) were found in amplified *EGFR* gene fragments, among which 1 was common in seven patient samples. On the other hand, twenty four different mutations (52.17%) were found in *PIK3CA* gene fragment amplicons, among which 2 were found in more than 1 patient. Four mutations (8.7%) were found in *KRAS* gene fragment amplified products. Our study shows that except for *KRAS*, the frequency of observed mutations in our patients is higher than those reported earlier in other parts of the world. The study can be used as a basis to build a mutation database for cervical cancer in Bangladesh. With the possibility of targetable oncogenic mutations, further exploration can be oriented towards establishing future diagnostics, personalized medicine decisions, and other pharmaceutical applications for specific cancer subtypes.

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LIST OF ABBREVIATIONS

ACS	American Cancer Society
AJCC	American Joint Committee on Cancer
AML	Acute Myeloid Leukemia
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CBC	Complete Blood Count
CIN	Cervical Intraepithelial Neoplasia
CML	Chronic Myeloid Leukemia
CT	Computed Tomography
DES	Diethylstilbestrol
EBV	Epstein Barr Virus
ECC	Endocervical Curettage
ECG	Electro Cardiogram
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FIGO	International Federation of Gynecology and Obstetrics
GAVI	Global Alliance for Vaccines and Immunization
GDP	Guanosine diphosphate
GMT	Geometric Mean Antibody Titers
GTP	Guanosine triphosphate
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HHV8	Human Herpesvirus 8
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HTLV-1	Human T-Lymphotropic Virus Type 1
IMRT	Intensity-Modulated Radiation Therapy
KRAS	Kirsten Rat Sarcoma
LEEP	Loop Electrosurgical Excision Procedure
MCPyV	Merkel Cell Polyomavirus

MRI	Magnetic Resonance Imaging
MTBE	Methyl tert-butyl ether
NCBI	National Center for Biotechnology Information
NCCN	National Comprehensive Cancer Network
NHS	National Health Service
NICHR	National Institute of Cancer Research and Hospital
NIH	National Institute of Health
OCs	Oral Contraceptives
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFOA	Perfluorooctanoic acid
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PIK3CA	Phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha
PtdIns	Phosphatidylinositols
SC	Squamocolumnar
TNM	Tumor Node Metastasis
TSG	Tumor Suppressor Genes
US FDA	United States Food and Drug Administration
VEGF	Vascular Endothelial Growth Factor
VIA	Visual Inspection With Acetic Acid
VLPs	Virus Like Particles
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Cervical cancer is a type of gynecologic cancer that develops in the cervix, which is a part of the uterus. Worldwide, cervical cancer was the fourth most common cancer among females in 2012 (World Cancer Report 2014). There was a report estimating 528,000 new cases of cervical cancer, of which around 85% occurred in less developed regions (World Cancer Report 2014). Around 266,000 deaths occur due to cervical cancer, accounting for 8% of all female cancer deaths (World Cancer Report 2014). In 2014, an estimated 12,578 women in the United States were diagnosed with cervical cancer; among them, 4115 women died of the disease (CDC; https://www.cdc.gov/cancer/cervical/statistics/index.htm). The widespread use of cervical cancer screening programs has dramatically reduced rates of cervical cancer in developed countries (Canavan and Doshi 2000).

Infection with HPV is the most important risk factor for cervical cancer (Snijders, Steenbergen et al. 2006). In humans, specific papillomavirus types have been associated with over 99% of cervical cancer biopsies (Walboomers, Jacobs et al. 1999). It is a common virus that can be sexually transmitted. Sexually active women commonly harbor HPV infection which is highly contagious to their partner (NHS Choices, 2013). HPV belongs to papillomavirus family and is a small, icosahedral, non-enveloped DNA virus (Bosch, Manos et al. 1995). HPV show tropism for stratified squamous epithelium. More than 200 genotypes of HPV have been found and were classified into two groups named the oncogenic and the non-oncogenic group (Bosch, Lorincz et al. 2002). The oncogenic group is again subdivided into two classes: high risk and low-risk group. Only 15 are identified as high-risk types. They can cause neoplastic changes to the cervical epithelium. Globally, 75% of cervical cancer cases are caused by HPV types 16 and 18, while 31 and 45 are the causes of another 10% (Oldham 2009).

Besides HPV, multiple factors such as cigarette smoking (both active and passive), long-term use of oral contraceptives, multiple pregnancies, low socioeconomic status, being immunocompromised, multiple sexual partners are associated with increased risk of cervical cancer (Murray, Rosenthal et al. 2015).

The risk of cancerous or precancerous lesion of the cervix and perineum is reduced with the use of two HPV vaccines named Gardasil and Cervarix which are effective between

92% and 100% against HPV 16 and 18 up to at least 8 years. The vaccines minimize the risk by about 93% and 62% respectively (Medeiros, Rosa et al. 2009).

Cervical cancer, like other cancers, is a disorder of tissue growth regulation. Different type of genetic changes that can occur in a cell includes aneuploidy, polymorphism, mosaicism, mutation etc. Mutation is a common change, which means any permanent changes in genomic DNA. Mutation may be missense, nonsense, insertion, deletion, duplication, frameshift mutation and repeat expansion (Fagundes, Perez, Grigsby, & Lockett, 1992). These mutations are considered as significant when they occur in genes which control different signal transduction pathways. This is because these pathways exert important biological and cellular functions including cell growth, cell cycle control, cell differentiation, apoptosis etc. There are specific genes which regulate cell growth and differentiation. Altered function of these genes leads a normal cell to transform into a cancer cell (Croce 2008).

Among the high number of genes involved in different signal transduction and cell growth regulation pathways, some are of special interest. Mutations in these genes play a key role in the development of different cancers. These genes are *EGFR* (Epidermal Growth Factor Receptor), *KRAS* (Kirsten rat sarcoma), and *PIK3CA* (phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha). The protein product of *EGFR* gene is a receptor for members of the epidermal growth factor family (EGF family) of extracellular protein ligands (Herbst 2004). *KRAS* gene which is a proto-oncogene corresponding to the oncogene that was first identified in Kirsten rat sarcoma virus (Tsuchida, Ohtsubo et al. 1982) and its protein product is a GTPase that is an early player in many signal transduction pathways. Protein product of *PIK3CA* (phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha) gene uses ATP to phosphorylate phosphatidylinositols (PtdIns), PtdIns4P and PtdIns P2.

The aim of this project is to find out mutation of any of these genes in cancerous tissue of cervical carcinoma patients in Bangladesh and to rule out the significance of these mutations in developing the disease as well.

1.2 Literature review

1.2.1 Cancer

Cancer is a complex disease that is the result of different types of genetic and epigenetic alterations. Neoplasm is the term implies for abnormal cell growth. The eminent British oncologist Willis defined neoplasm: "A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change." Neoplasm is of two types: benign and malignant. Malignant neoplasm is generally known as cancer. Neoplasm is called tumor when it forms a lump or growth. A malignant tumor or cancer is different from a benign tumor in a way called metastasis, which means spread of cancer cell from the original (primary) tumor through the blood or lymph system, and formation of a new tumor (secondary) in other organs or tissues of the body. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements (NHS). However, these symptoms are caused by other diseases also (NHS).

1.2.1.1 Causes of cancer

Smoking and tobacco, diet and physical inactivity, sun and other types of radiation, viruses are recognized as most common causes of cancer (ACS). Some percentage of cancer cases are inherited. Other causes are environmental factors which mean any cause that is not inherited genetically, such as lifestyle, economic and behavioral factors and not merely pollution (Kravchenko *et al*., 2009).

Carcinogenesis is a term implying the development of cancerous cells from normal ones and environmental causes of cancer fall into the following five categories: a) chemical carcinogenesis, b) radiation carcinogenesis, c) microbial carcinogenesis, d) nutritional carcinogenesis, and e) hormonal carcinogenesis. In the perspective of increasing exposures to different types of chemicals and radiation, possible and known environmental causes of cancer include acrylamide, agent orange, alcohol, antiperspirants, arsenic, asbestos, aspartame, cellular towers, cosmetics, diesel exhaust, formaldehyde, hair dyes, lead, medical treatments, microwaves and radio waves, MTBE, power lines and electrical devices, radon, recombinant bovine growth hormone,

smart meters, talcum powder, teflon and perfluorooctanoic acid (PFOA), tetraclorethylene (perchloroethylene), UV radiation (sun), water fluoridation, x-rays and gamma rays (ACS). Further, chemical carcinogens are of two types: directly acting compound and indirectly acting compounds (or pro-carcinogens). Direct acting compounds are again subdivided into alkylating agents and acylating agents. Examples of alkylating agent include β-propiolactone, dimethyl sulfate, diepoxybutane, anticancer drugs (cyclophosphamide, chlorambucil, bleomycin, nitrosoureas and others). 1-acetyl-imidazole, dimethylcarbamyl chloride are acylating agents. Procarcinogen is a substance that is not directly carcinogenic but can be converted into a carcinogen by metabolic processes in the body. Examples of procarcinogens are polycyclic aromatic hydrocarbons, aromatic amines, amides and azo dyes.

Microbes like virus, bacteria, and fungus can be oncogenic and may induce neoplastic transformation in the cell. Hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), Epstein Barr virus (EBV), human herpesvirus 8 (HHV8), Merkel cell polyomavirus (MCPyV), and human T-lymphotropic virus type 1 (HTLV-1) virus are oncogenic in human (Luo & Ou, 2015). *Helicobacter pylori* are well known oncogenic bacteria which can cause gastric cancer (Chang & Parsonnet, 2010). Overconsumption of energy, obesity, consumption of red meat, imbalance of omega 3 and omega 6 fats, low fiber intake can contribute to nutritional carcinogenesis (Donaldson 2004). It has been estimated by the American Institute for Cancer Research and the World Cancer Research Fund that 30–40 percent of all cancers can be prevented by appropriate diets, physical activity, and maintenance of appropriate body weight (Potter 1997). Hormones, both endogenous and exogenous, induce random genetic errors by driving cell proliferation, increased the number of cell divisions. Hormone replacement therapy and oral contraceptives are two forms of exogenous hormones and one or both of these agents play a role in the risk of breast, ovary, cervical, endometrial and colorectal cancers (Henderson and Feigelson 2000). Common environmental factors that contribute to cancer death include tobacco (25–30%), diet and obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity and pollution (Anand, Kunnumakara et al. 2008). Eventually, all the agents cause genetic change and induce neoplastic transformation of cells.

1.2.1.2 Cancer genetics

Proto-oncogenes and tumor-suppressor genes are two main classes of a gene that regulate cell growth and differentiation. Alteration of the function of these genes can cause transformation normal cell to cancer cell (Croce 2008). The activation of oncogenes or inactivation of tumor suppressor genes (TSG) results in genetic and/or epigenetic changes that lead a normal cell into a neoplastic cell. A set of phenotypic characteristics occur in this neoplastic cell that provides a growth advantage on the cell for survival in the tumor microenvironment. A proto-oncogene is a normal gene that regulates cell growth and differentiation by signal transduction pathway and execution of mitogenic signal, but could become a tumor-inducing agent, an oncogene due to mutations or increased expression (Figure 1.1).

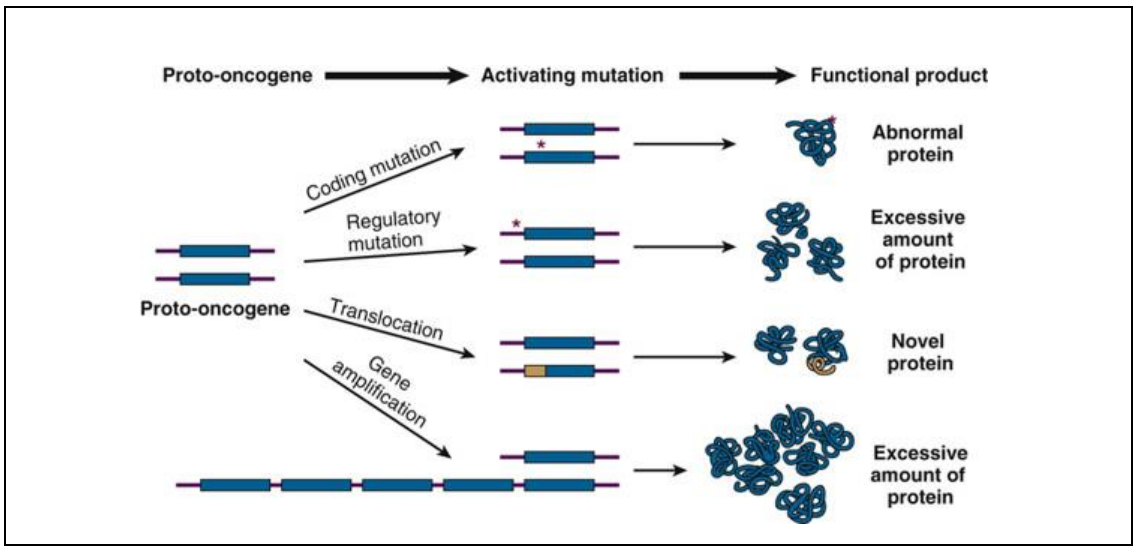

Figure 1.1: Functions of proto-oncogene. Source: (Key, 2016).

The proteins coded by tumor-suppressor genes, either have a dampening or repressive effect on the regulation of the cell cycle or promote apoptosis and sometimes do both (Figure 1.2). Typically, changes in multiple genes are required to transform a normal cell into a cancer cell (Knudson 2001).

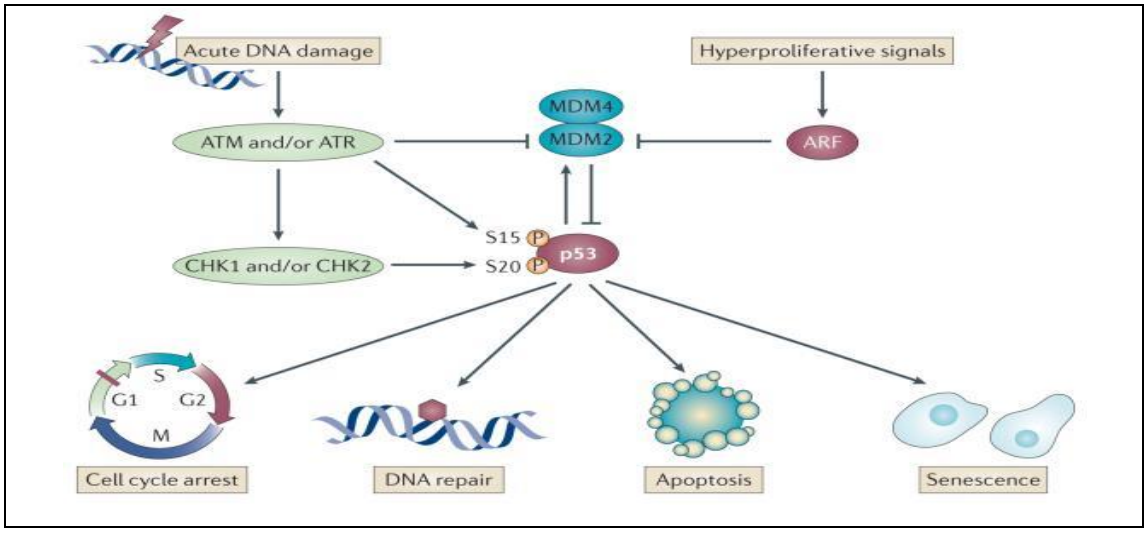

Figure 1.2: Functions of tumor suppressor genes. Source: (Bieging, Mello, & Attardi, 2014).

Genetic changes most commonly occur through mutation. Large-scale mutations involve the deletion or gain of a portion of a chromosome. Genomic amplification occurs when a cell gains copies (often 20 or more) of a small chromosomal locus, usually containing one or more oncogenes and adjacent genetic material. Translocation occurs when two separate chromosomal regions become abnormally fused, often at a characteristic location. Small-scale mutations include point mutations, deletions, and insertions, which may occur in the promoter region of a gene and affect its expression, or may occur in the gene's coding sequences and alter the function or stability of its protein product. Disruption of a single gene may also result from the integration of genomic material from a DNA virus or retrovirus, leading to the expression of viral oncogenes in the affected cell and its descendants.

The transformation of a normal cell into a cancerous cell is akin to a chain reaction caused by initial errors, which compound into more severe errors, each progressively allowing the cell to escape more controls that limit normal tissue growth.

1.2.1.3 Hallmarks of cancer

Six basic acquired capabilities that confer cells with growth advantages in the tumor microenvironment are shared amongst all cancers (Hanahan and Weinberg 2000). These six hallmarks are i) sustaining proliferative signaling, ii) evading growth suppressors, iii) activating invasion and metastasis, iv) enabling replicative immortality, v) inducing angiogenesis and vi) resisting cell death (Figure 1.3). To

acquire the hallmarks, two enabling characteristics are required which were proposed later: a) genome instability and mutation and b) tumor-promoting inflammation. Two more emerging hallmarks are added in a follow-up manuscript named c) deregulating cellular energetics and d) avoiding immune destruction (Hanahan and Weinberg 2011).

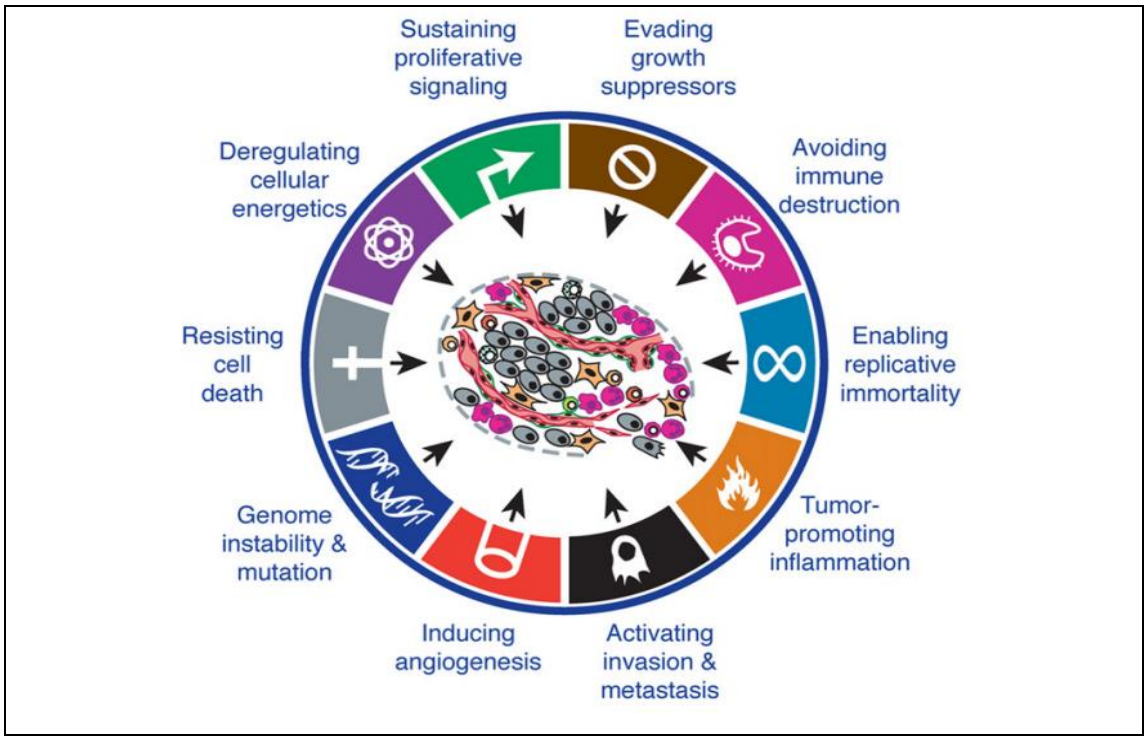

Figure 1.3: Hallmarks of cancer. Most if not all cancers acquire the same set of characteristics during their progression through various strategies. Modified from (Hanahan and Weinberg 2011).

1.2.1.4 Phases of carcinogenesis

Carcinogenesis phases are initiation, promotion and progression (Figure 1.4) (DiGiovanni, 1992). Initiation involves the alteration, change, or mutation of genes arising spontaneously or induced by exposure to a carcinogenic agent. Genetic alterations can result in dysregulation of biochemical signaling pathways associated with cellular proliferation, survival, and differentiation, which can be influenced by a number of factors, including the rate and type of carcinogenic metabolism and the response of the DNA repair function. The promotion stage is considered to be a relatively lengthy and reversible process in which actively proliferating preneoplastic cells accumulate. Within this period, the process can be altered by chemopreventive agents and affect growth rates. Progression is the phase between a premalignant lesion and the development of invasive cancer. Progression is the final stage of neoplastic

transformation, where genetic and phenotypic changes and cell proliferation occur. This involves a fast increase in the tumor size, where the cells may undergo further mutations with invasive and metastatic potential. Chemopreventive agents should be able to preferentially act within the initiation and promotion processes of carcinogenesis.

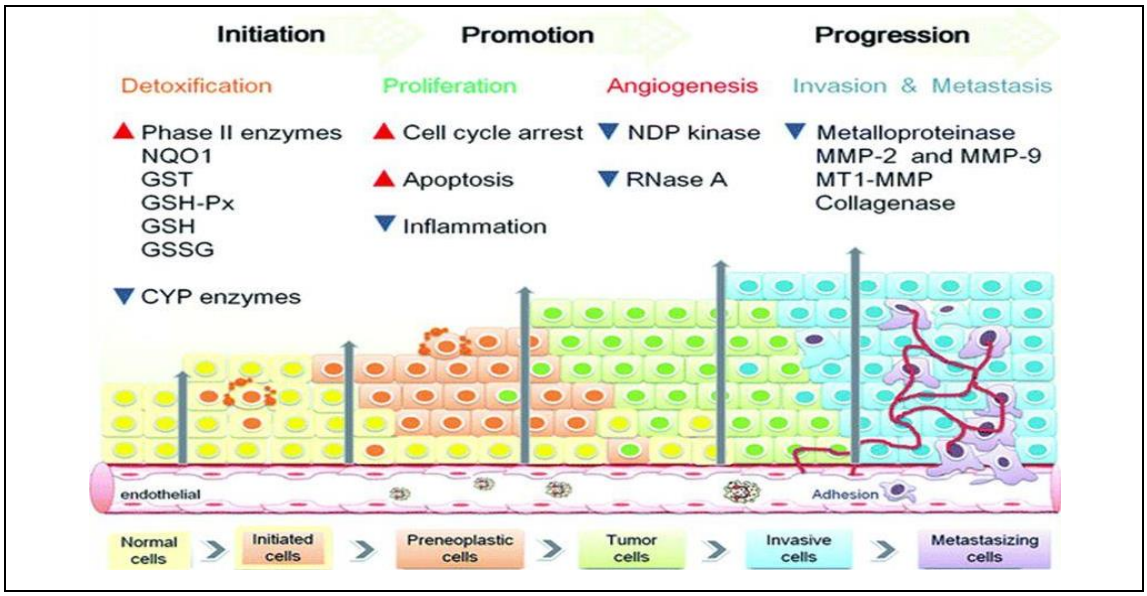

Figure 1.4: Phases of carcinogenesis. Source (Pan, Chiou et al. 2011).

1.2.1.5 Global statistics of cancer

Cancer with an incidence of approximately 14 million new cases in 2012 is one of the leading causes of morbidity and mortality worldwide (Ferlay et al., 2015) and the number of new cases is expected to rise by about 70% over the next 2 decades. Total 8.8 million deaths occurred due to cancer in 2015. Globally, nearly 1 in 6 deaths is due to cancer (WHO). Approximately 70% of deaths from cancer occur in low- and middle-income countries. High body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use account for around one-third of deaths from cancer. Tobacco use is responsible for approximately 22% of cancer deaths and recognized as is the most important risk factor for cancer (Forouzanfar et al., 2015). Cancer causing infections, such as hepatitis and human papilloma virus (HPV), are responsible for up to 25% of cancer cases in low- and middle-income countries (Plummer et al., 2016). Less than 30% of low-income countries reported treatment services are available to public sector compared to More than 90% of high-income countries. The economic impact of cancer is significant and is increasing. The total annual economic cost of cancer in 2010 was approximately US$ 1.16 trillion (World

Cancer Report, 2014). Only 1 in 5 low- and middle-income countries have the necessary data to drive cancer policy (GICR).

1.2.2 Cervical cancer

1.2.2.1 Anatomy of the cervix

1.2.2.1.1 Gross anatomy of the cervix

Uterus, which is the major organ of the internal genital system of females, has 3 parts, i.e., fundus, body, and cervix. Cervix occupies the lower 1/2 to 1/3rd length of the uterus. Cervix is 2.5 to 3.0 cm long, 2.0 to 2.5 cm in diameter and cylindrical in shape. It connects the body of the uterus (corpus uteri) to vagina via the endocervical canal. Anteriorly it is separated from the bladder by connective tissue; posteriorly is covered by peritoneum that forms the lining of cul-de-sac.

The cervix has following parts (Figure 1.5):

Endocervix: relates to the endocervical canal, fusiform, 7-8 mm in diameter, with anterior and posterior longitudinal and oblique mucosal ridges (plicae palmatae/arbor vitae uteri).

Ectocervix (exocervix): vaginal portion of the cervix, convex, flattened anterior-posteriorly, has anterior and posterior lips, is surrounded by fornix (a reflection of vaginal wall).

External os: the opening of endocervical canal into vagina, round in nullipara and slit-like in parous women.

Internal os: the opening of endocervical canal into uterine cavity.

Fornix: recesses of deeper portions of vagina created by protrusion of ectocervix into the vagina; anterior, posterior and a lateral fornix on either side of the cervix.

Cardinal / Mackenrodts / Lateral cervical ligaments: fibromuscular bands that fan out from lower uterine segment and cervix to lateral pelvic walls and provide the main support for cervix.

Ligaments: connective tissue surrounding cervix and vagina that extends towards vertebrae.

Blood vessels: cervix is supplied by branches of uterine artery. Veins of the cervix drain into uterine vein.

Lymphatics: cervix is drained by parametrial, internal iliac, obturator and presacral nodes (Konar 2016).

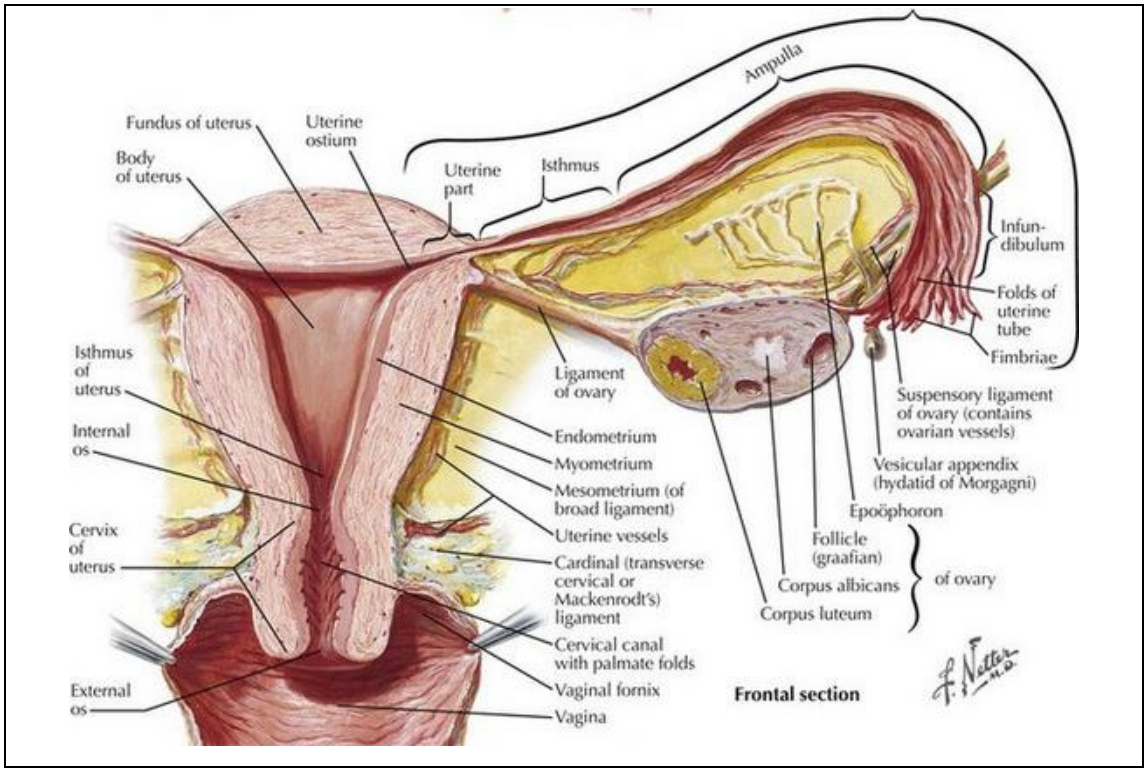

Figure 1.5: Anatomy of the cervix. Source: (Netter, 2014).

1.2.2.1.2 Histology of cervix

Endocervix is lined by simple columnar epithelium while ectocervix is lined by stratified squamous epithelium (Figure 1.6). There is an area called squamocolumnar (SC) junction where squamous and columnar epithelium meets. Transformation zone (also called ectropion) is the area between original SC junction and new SC junction due to regenerative metaplastic response; which is the site of >90% of squamous cell carcinomas and dysplasia.

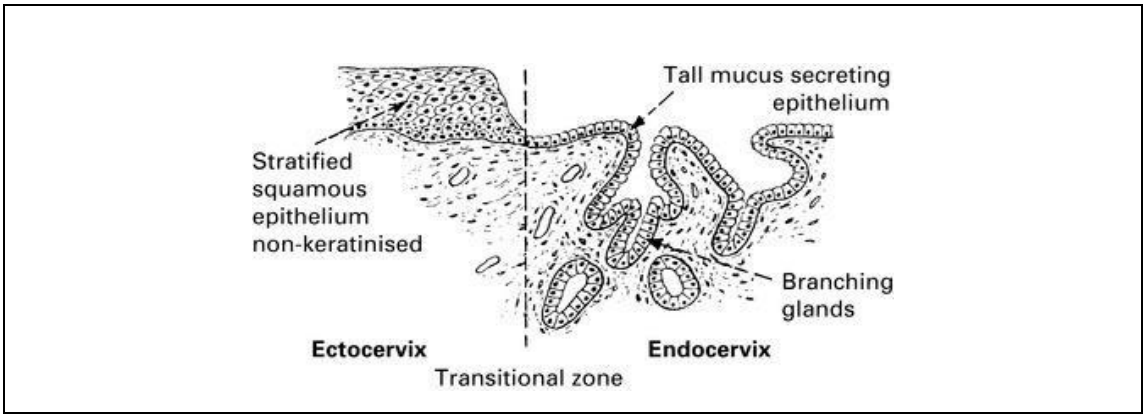

Figure 1.6: Histology of cervix. Source: (Iknowledge, 2015).

1.2.2.2 Premalignant condition of the cervix

The premalignant condition of the cervix includes cervical lesions that eventually lead to cervical cancer if untreated. Examples are basal cell hyperplasia, squamous cell metaplasia, leukoplakia, cervical dysplasia and cervical intraepithelial neoplasia (CIN). Various screening tests are available that can detect these pre-cancerous conditions and thus cervical cancer can be prevented.

1.2.2.2.1 Cervical Intraepithelial Neoplasia

Among these conditions, CIN which is very common is a histological observation where part or whole of the thickness of the cervical epithelium is replaced by varying degree of atypical cells (Dutta and Konar 2016).

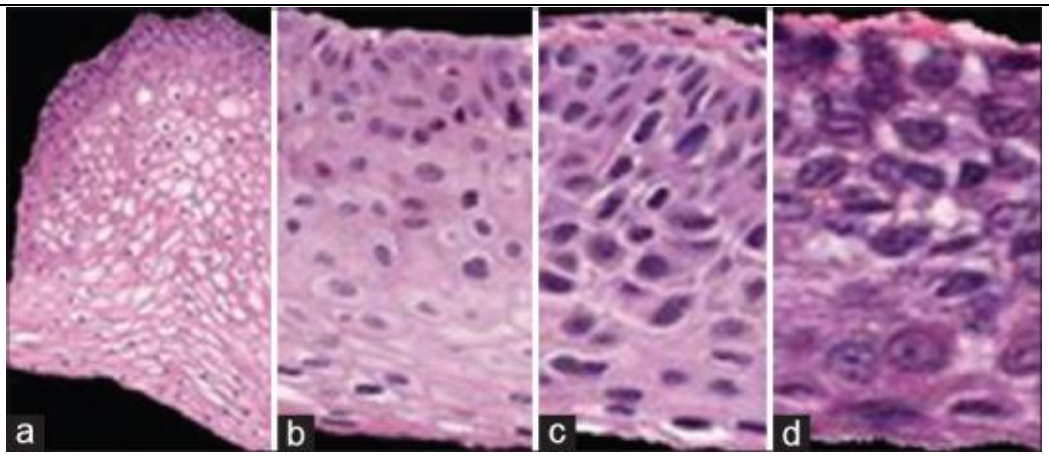

Figure 1.7: Cervical intraepithelial neoplasia grade label examples highlighting the increase of immature atypical cells from epithelium bottom to top with increasing cervical intraepithelial neoplasia severity. (a) Normal, (b) cervical intraepithelial neoplasia 1, (c) cervical intraepithelial neoplasia 2, (d) cervical intraepithelial neoplasia 3. Source: (Guo et al., 2016).

1.2.2.3 Tumors of the cervix

Cervical tumors are classified as benign and malignant tumors. Papilloma, adenofibroma, angioma, fibromyoma, myoma are the benign tumors of the cervix. 90% of the malignant tumors of the cervix (cervical cancers) are squamous cell carcinomas (Jeffcoate 1954). Other 5 to 10% cancers are adenocarcinomas. Cervical cancers having features of both squamous cell carcinomas and adenocarcinomas are called adenosquamous carcinomas or mixed carcinomas (ACS). There are some rare varieties of cervical cancers, e.g., adenoma malignum, adenocanthoma, mesonephric tumor, clear cell tumor, mucoepidermoid tumor, glassy cell carcinoma, etc.

1.2.2.4 Gross Appearance of cervical cancer

There are three categories of gross appearance of cervical carcinoma (Figure 1.8):

1) Exophytic lesions: Most common form and arises on ectocervix. Grows to form large, friable, polypoidal masses that bleed profusely.

2) Infiltrating lesions: Presents as stony hard cervix with a minimal or invisible lesion on the cervix.

3) Ulcerative lesions: Presents as an ulcer over the cervix, often replacing the whole of the cervix.

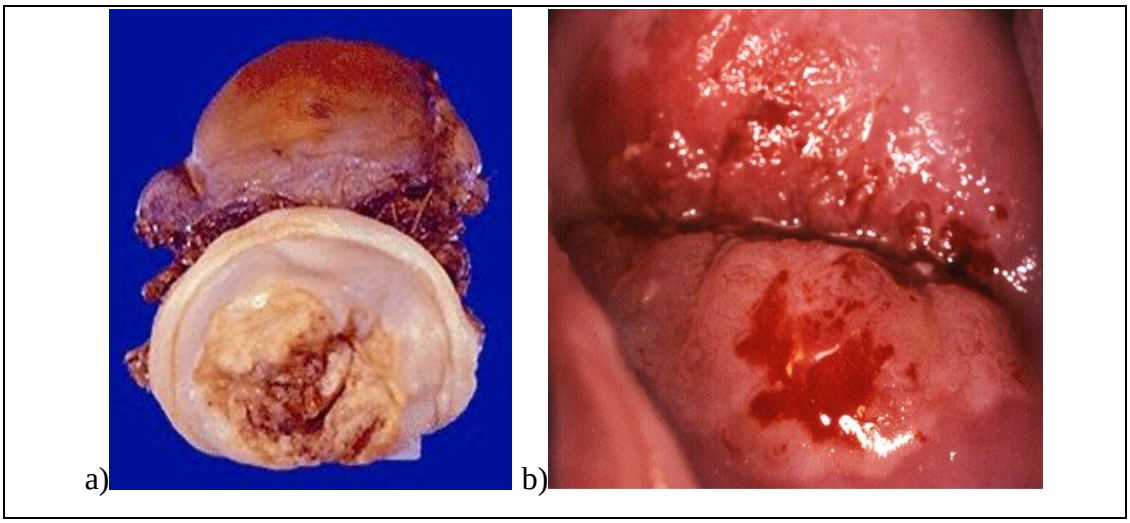

Figure 1.8: Gross Appearance of cervical cancer. a) Exophytic variety (WebPath, 2018) and b) Ulcerative type (Sankaranarayanan & Wesley, 2003).

1.2.2.5 Dissemination and Spread

Like other cancer of epithelial origin, cervical carcinoma metastasizes mainly through lymphatic route. Direct local extension is also a common form of metastasis of cervical cancer. Unusual types of cervical cancer, such as adenosquamous or neuroendocrine tumors disseminates through hematogenous route. Blood spread occurs commonly also in advanced disease.

1.2.2.6 Causes and risk factors for cervical cancer

In recent years, there has been a lot of progress in understanding what happens in cells of the cervix when cancer develops. In addition, several risk factors have been identified that increase the odds that a woman might develop cervical cancer.

1.2.2.6.1 Human Papillomavirus (HPV)

HPV is a DNA virus from the papilloma viridae family. HPV like other members of the family favors the higher vertebrates as host and is very species-specific too. More than 200 genotypes of HPV have been recognized and were classified into two groups - the oncogenic and non-oncogenic group (Bosch, Lorincz et al. 2002). A study shows that 15 HPV types are high-risk types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) 3 are probable high-risk types (types 26, 53 and 66) and 12 classified as low-risk types (types 6, 11, 440, 42, 43, 44, 54, 64, 70, 72, 81 and Cp6108) (Muñoz, Bosch et al. 2003). Usually, HPV affects the skin and the moist membranes lining body Including cervix, anus, mouth, and throat (NHS choice). Genital HPV infections are common and highly contagious. They are spread during sexual intercourse and skin-to-skin contact of the genital areas (NHS choice).

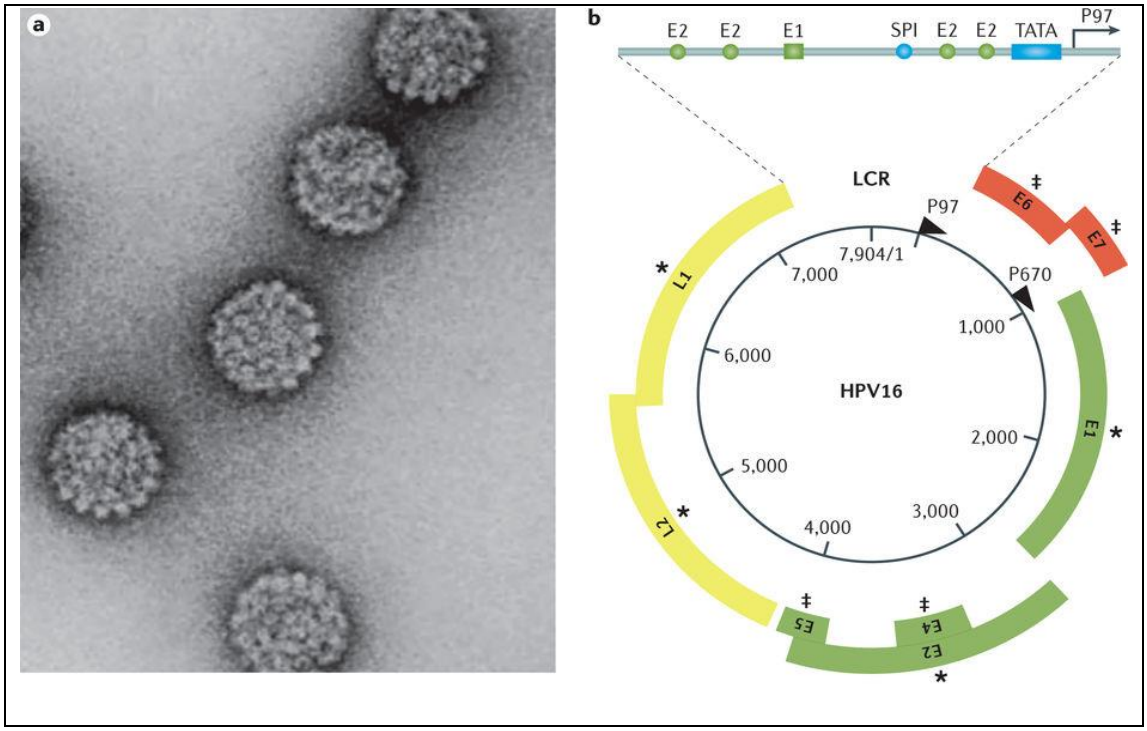

Figure 1.9: Human Papilloma Virus. a) Human papillomavirus (HPV) particles (55 nm in diameter) are shown in the negatively stained transmission electron micrograph. b) The genome organization typical of the high-risk Alpha HPV types illustrated as HPV16. Source: (Schiffman et al., 2016).

1.2.2.6.1.1 Disease caused by HPV

Molecular and epidemiological evidence has now established that HPV types associated with anogenital neoplasms, including condylomata, cervical dysplasia, and cervical carcinoma, are almost always sexually transmitted (Lowy, Kirnbauer et al. 1994). HPV causes genital warts, which are a form of benign tumor of epithelial cells. Various serotypes are responsible for genital warts and usually not related to cervical cancer. It is common to have an infection with multiple strains at the same time, including those that can cause cervical cancer along with those that cause warts. The involvement of HPV in cancers of the vulva, anal canal, vagina, and penis is currently being identified. In addition to these, the possible infectivity of HPV in cutaneous cancer, oral cancers and other cancers of the upper aerodigestive tract is being investigated (Bosch, Lorincz et al. 2002).

1.2.2.6.1.2 Cervical cancer and HPV

Human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease in both men and women worldwide (Burd 2003). Ninety-five percent of cervical cancer cases are caused by persistent infections with carcinogenic HPV (Schiffman, Wentzensen et al. 2011). Infection with HPV may be latent, subclinical, or clinical. It may take the pathway of low viral load infection without clinical disease, or high viral load infection with clinical disease. HPV tends to follow the iceberg concept of pathogenesis, and as such Most HPV infections are transient and asymptomatic, resolving typically within 12 to 18 months. Nevertheless, on rare occasions, latent or persistent infections may be established by the consistent maintenance of HPV genome in episomal form. This persistence, particularly if caused by certain high-risk HPV types, is a necessary condition for the development of cervical cancer (ACS).

1.2.2.6.1.3 Progression of cervical cancer by HPV infection

Unlike many genitourinary infections HPV infection, is not usually presented with immediate symptoms such as itching, burning, and vaginal discharge (Mao, Hughes et al. 2003). Rather, due to host defense, the majority of HPV infection will not progress to clinical disease or symptoms. The exact mechanism by which HPV infection is cleared by the host immune system is currently unknown. A study shows genital warts developed only in 24.8% of women infected with HPV 6 or 11 (Mao, Hughes et al. 2003). A large, prospective 10-year cohort study of more than 20,000 women enrolled in a health maintenance organization found that approximately 7% in HPV-positive women developed CIN 3 or cancer (Sherman, Lorincz et al. 2003).

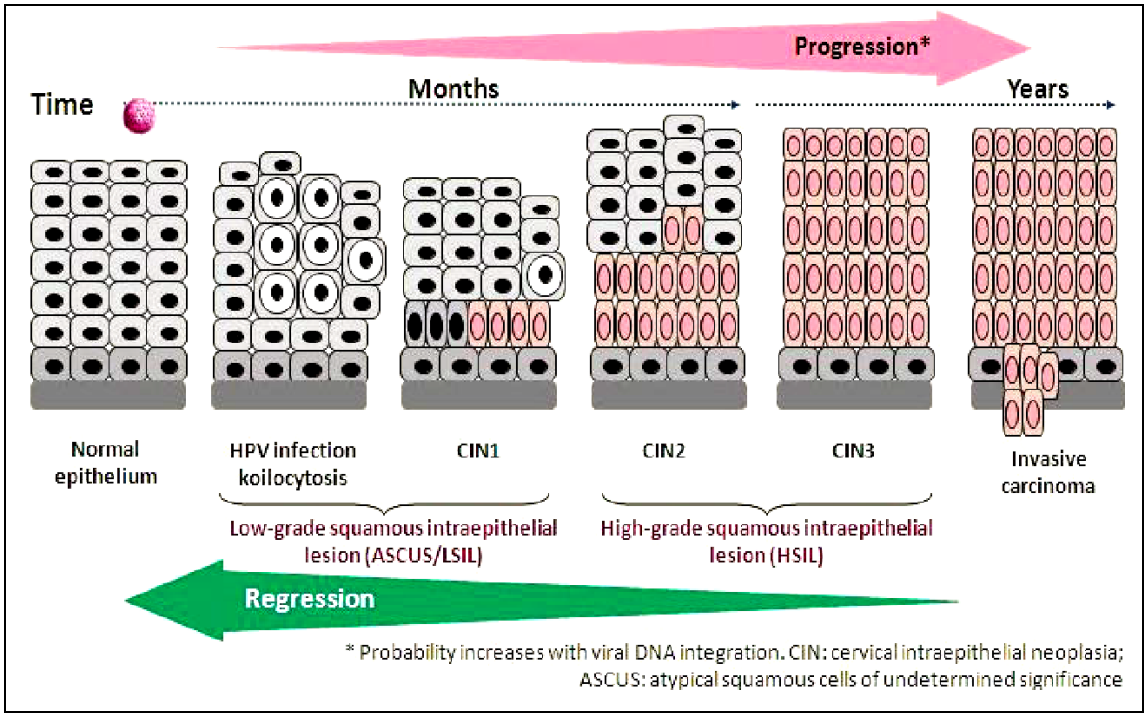

Figure 1.10: Progression of cervical cancer with HPV infection. Source: (Burd 2003)

1.2.2.6.2 Age

Age was a significant prognostic factor for cervical cancer (Brun, Stoven-Camou et al. 2003). Prognosis is worse with the increase of age. However, cervical cancer tends to occur in midlife. Most cases are found in women younger than 50. Women younger than 20 rarely develops cervical cancer. Around15% of cases of cervical cancer are found in women over 65 (ACS).

1.2.2.6.3 Early exposure to sexual intercourse or early marriage

Girls exposing to sexual intercourse in their early teen ages have more chance to develop cervical cancer. It is probably because of vulnerability to HPV infection of young girls.

1.2.2.6.4 Early pregnancy

Women having their first full-term pregnancy below the age of 17 years are in two times higher risk for developing cervical cancer later in life than women who get pregnant after the age of 25 years (Green, De Gonzalez et al. 2003).

1.2.2.6.5 Multiple pregnancies

Multiple births are an important risk factor for cervical cancer. Chance of developing cervical cancer is more with women who have had 3 or more full-term pregnancies. The exact reason behind this remains unknown. One hypothesis is that these women have frequent and more unprotected intercourse to get pregnant, so they may have had more exposure to HPV. Another prediction is that immunosuppression during pregnancy makes women more susceptible to HPV infection.

1.2.2.6.6 Multiple sexual partners

Men or women having multiple sexual partners easily get infected with HPV (Konar 2016).

1.2.2.6.7 An immunocompromised status

An important risk factor for persistent infections with human papillomaviruses (HPV) and HPV-associated disease is chronic immunosuppression caused by various conditions (Wieland, Kreuter, & Pfister, 2014). Conditions such as Human immunodeficiency virus (HIV) infection, diabetes mellitus, use of chemotherapy, steroid or immunosuppressive drug, chronic kidney disease, malnutrition, protein losing enteropathy, rheumatoid arthritis, multiple myeloma etc. are associated with immunosuppression and people having any of these conditions are prone to HPV infection.

1.2.2.6.8 Smoking

When someone smokes, they and those around them are exposed to many cancer-causing chemicals that affect extra-pulmonary organs as well. Women who smoke are about twice as likely as non-smokers to get cervical cancer. Tobacco by-products have been found in the cervical mucus of women who smoke. Factors that cause a neoplastic change in cervix in smoker women are direct local carcinogenic effect and local immunosuppression (Fonseca-Moutinho, 2011). Passive smoking is also associated with increased risk, but to a lesser extent (NIH).

1.2.2.6.9 Low socioeconomic status

Low socioeconomic status is associated with unhygienic living. Women of these families cannot maintain adequate sanitation during menstruation. This condition makes women of this family vulnerable to HPV infection. They also do not have easy access to adequate health care services, including Pap tests. This means they may not get screened or treated for cervical pre-cancerous conditions.

1.2.2.6.10 *Chlamydia* infection

Chlamydia is a common bacterium that can infect the urogenital system and is transmitted by sexual contact. Studies have shown that risk of cervical cancer is higher in women having past or current *Chlamydia* infection in cervix or blood (Chang & Parsonnet, 2010).

1.2.2.7 Dietary habits

A diet deficient in fruits, vegetables, as well as being overweight, increases the risk of cervical cancer (ACS).

1.2.2.7.1 Long-term use of oral contraceptives

Risk of the cervical cancer is increased with long time taking of oral contraceptives (OCs). Studies have shown that the longer the use of OCs, the greater the risk of cervical cancer (NIH).

1.2.2.7.2 Diethylstilbestrol (DES)

DES increases the risk of adenocarcinoma in the cervix, especially in women whose mothers took DES when pregnant (ACS).

1.2.2.7.3 Positive family history

Cervical cancer may run in families. If mother or sister or first-degree female relatives of a woman have cervical cancer, her chance of developing the disease is more. Some researchers predict familial tendency to develop cervical cancer is associated with an inherited condition that makes women vulnerable to HPV infection than others.

1.2.2.8 Signs and symptoms

Abnormal vaginal bleeding which may be irregular or continued in the form of intermenstrual bleeding, contact (postcoital) bleeding or post-menopausal bleeding is a cardinal symptom. The patient may present with offensive vaginal discharge, pelvic pain of varying degree, leg edema and anemia. In advanced disease, metastases to bladder, rectum, pelvic wall, other pelvic organ and distantly to lungs or elsewhere can occur. In case of bladder involvement, the patient may have complaints of the frequency of micturition, dysuria, hematuria, true urinary incontinence. Features such as diarrhea, rectal pain, bleeding per rectum, rectovaginal fistula indicate rectal involvement (POTTER 1997). General Symptoms and signs of cervical cancer include cachexia, pallor, loss of appetite, weight loss, fatigue, uremia (Jeffcoate1954). However, the early stages of cervical cancer may be completely free of symptoms (Canavan and Doshi 2000).

1.2.2.9 Diagnosis

Available tests for cervical cancer diagnosis are Pap test, colposcopy, and cervical biopsy. Pap test is a screening test and may be done primarily in suspicious cases. But it is false negative in up to 50% of cases of cervical cancer (Figure 1.11) (Berek and Hacker 2010). Colposcopy is a magnified visual inspection of the cervix aided by using a dilute acetic acid solution to highlight abnormal cells on the surface of the cervix (Kumar, Abbas et al. 2017). Colposcopy is recommended if Pap test result is abnormal. A sample of tissue (biopsy) is collected for laboratory testing (histopathology) when an unusual area of cells during the colposcopic examination is seen. Biopsy is the most important investigation in diagnosing cervical cancer (Petignat and Roy 2007). It is a confirmatory test for the diagnosis of cervical cancer which can be done by 3 methods:

- **Punch biopsy:** In this method, small pieces of tissue are taken from the cervix with an instrument called biopsy forceps. Cervix might be stained with a dye to make it easier to see any abnormalities.

- **Cone biopsy:** This surgery uses a scalpel or laser to remove large, cone-shaped pieces of tissue from the cervix.

- **Endocervical curettage (ECC):** During this procedure, cells are removed from the endocervical canal (the area between the uterus and vagina). This is done with a hand-held instrument called a curette.

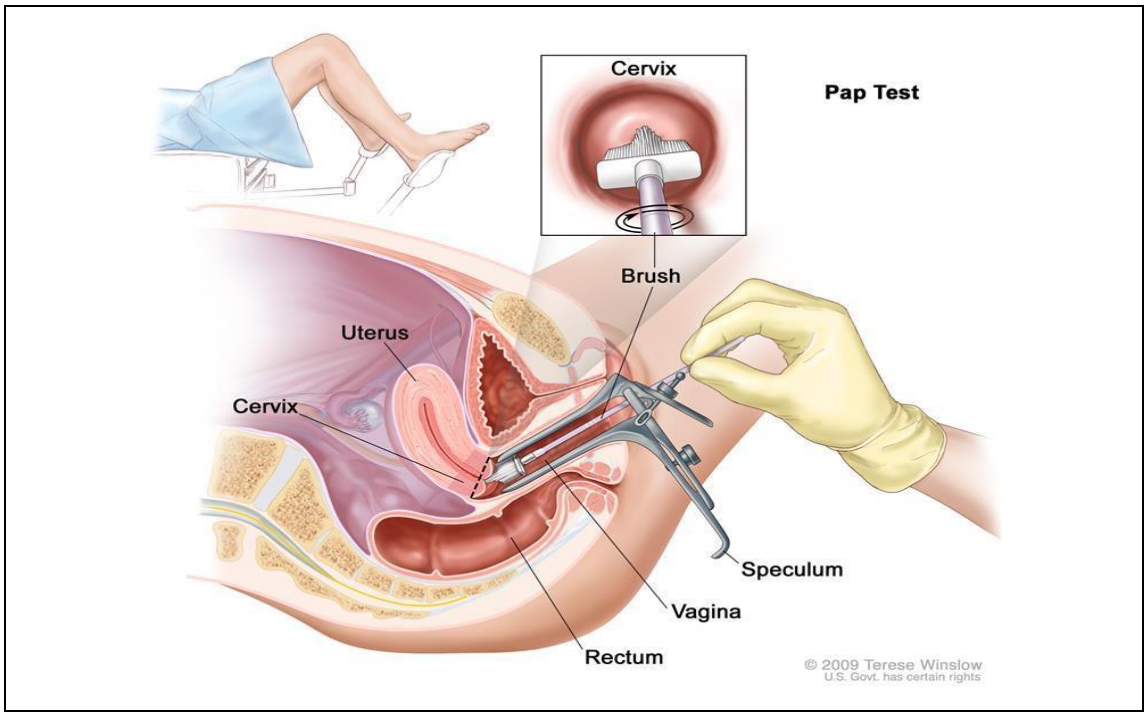

Figure 1.11: Pap test. Source: (ACS).

Once the diagnosis is confirmed, several other investigations including chest x-ray, CT scan of the whole abdomen, ultrasonography of the whole abdomen, colonoscopy, cystoscopy, intravenous pyelography, MRI, liver function test, renal function test are performed to determine the stage of cancer. General physical condition is assessed by CBC, serum electrolyte, ECG etc.

1.2.2.10 Cervical cancer stages

The different stages of cancer describe how far cancer has grown (tumor size) and spread at the time of diagnosis (metastasis). Information from clinical examination and tests is used to determine the size of a tumor, how deeply the tumor has invaded tissues in and around the cervix, and its spread to distant places (metastasis). The FIGO (International Federation of Gynecology and Obstetrics) staging system (Table 1.1) is most commonly used for cervical cancer. The stages are described using the number 0 and Roman numerals from I to IV.

The AJCC (American Joint Committee on Cancer) TNM staging system classifies cancer on the basis of 3 factors:

- The extent of the main tumor (T)
- Whether cancer has spread to nearby lymph nodes (N)
- Whether cancer has spread (metastasized) to distant parts of the body (M)

Information about the tumor (T), lymph nodes (N), and any cancer spread (M) is then combined to assign cancer an overall stage. This process is called stage grouping. The 5 years survival rates from the time patient presented to clinician of stage 1 is 85%, whereas the rate for stage 4 is 7% (Figure 1.12).

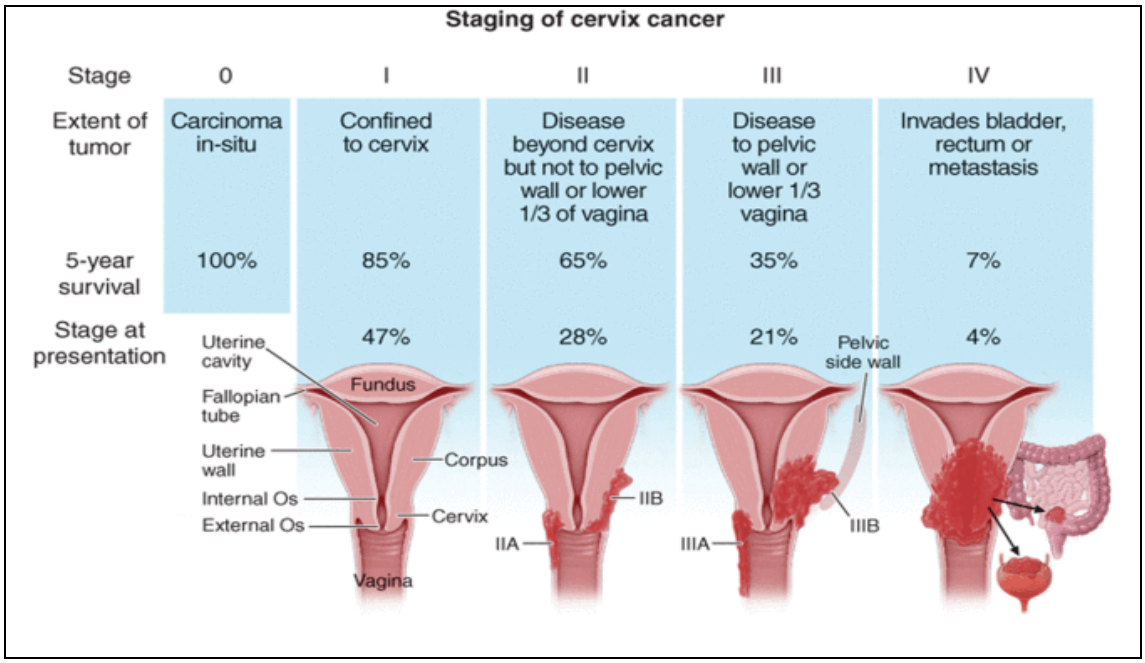

Figure 1.12: Stages of cervical cancer. Source: (Braunwald *et al.*, 2001).

Table 1.1: FIGO staging system of cervical cancer.

TNM staging *Categories*	**FIGO staging** *Stages*	**Surgical-Pathologic Findings**
Tx		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Tis		Carcinoma in situ (preinvasive carcinoma)
T1	I	Cervical carcinoma confined to the cervix (disregard extension to the corpus)
T1a	IA	Invasive carcinoma diagnosed only by microscopy; stromal invasion with a maximum depth of 5.0 mm measured from the base of the epithelium and a horizontal spread of 7.0 mm or less; vascular space involvement, venous or lymphatic, does not affect classification
T1a1	IA1	Measured stromal invasion ≤ 3.0 mm in depth and ≤ 7.0 mm in horizontal spread
T1a2	IA2	Measured stromal invasion > 3.0 mm and ≤ 5.0 mm with a horizontal spread ≤ 7.0 mm
T1b	IB	Clinically visible lesion confined to the cervix or microscopic lesion greater than T1a/IA2
T1b1	IB1	Clinically visible lesion ≤ 4.0 cm in greatest dimension
T1b2	IB2	Clinically visible lesion > 4.0 cm in greatest dimension
T2	II	Cervical carcinoma invades beyond uterus but not to pelvic wall or to lower third of vagina
T2a	IIA	Tumor without parametrial invasion
T2a1	IIA1	Clinically visible lesion ≤ 4.0 cm in greatest dimension
T2a2	IIA2	Clinically visible lesion > 4.0 cm in greatest dimension
T2b	IIB	Tumor with parametrial invasion

TNM staging *Categories*	**FIGO staging** *Stages*	**Surgical-Pathologic Findings**
T3	III	Tumor extends to pelvic wall and/or involves lower third of vagina and/or causes hydronephrosis or nonfunctional kidney
T3a	IIIA	Tumor involves lower third of vagina, no extension to pelvic wall
T3b	IIIB	Tumor extends to pelvic wall and/or causes hydronephrosis or nonfunctional kidney
T4	IV	Tumor invades mucosa of bladder or rectum and/or extends beyond true pelvis (bullous edema is not sufficient to classify a tumor as T4)
T4a	IVA	Tumor invades mucosa of bladder or rectum (bullous edema is not sufficient to classify a tumor as T4)
T4b	IVB	Tumor extends beyond true pelvis

1.2.2.11 Treatment

The main outcome of any cancer treatment is to preserve life and remove the tumor as quickly as possible. The options for treating cervical cancer depends on different factors including the type and stage of cancer, age of the patient, financial status, preserving reproductive function in case of a younger patient having desire to have children in future. According to type and stages, more than one type of treatment may be needed.

1.2.2.11.1 Treatment option for cervical cancer

1.2.2.11.1.1 Surgery

Early stages of cervical cancer are treated by surgery. Surgical options are diathermy, Conization, laser surgery, loop electrosurgical excision procedure (LEEP), trachelectomy, total abdominal hysterectomy, radical hysterectomy, exenteration. But surgery is the treatment of choice and curative only up to stage IB1

1.2.2.11.1.2 Radiation therapy

Irradiation is a treatment option for advanced cervical cancer. It may be external or internal. External radiation therapy Includes Intensity-modulated radiation therapy (IMRT). External-beam pelvic irradiation is usually given with a combination of intracavitary applications of a dose of 80Gy. Internal radiation therapy uses a radioactive substance sealed in needles, seeds, wires, or catheters that are placed directly into or near cancer.

1.2.2.11.1.3 Chemotherapy

As cervical cancer is mostly radiation sensitive, use of chemotherapy is limited to combination with radiotherapy and this type of intervention is called chemoradiotherapy.

1.2.2.11.1.4 Adjuvant therapy after radical surgery

- **High risk cases:** patients having nodal metastases with positive surgical margins are high risk cases. Treatment option after radical surgery is Adjuvant chemo-radiotherapy with external pelvic radiation along with weekly Cisplatin chemotherapy.
- **Intermediate risk:** cases are defined as an intermediate risk when cervical cancer is presented with the deep invasion of cervical stroma, parametrial extension, lymphovascular space invasion. Only radiation therapy is required after radical surgery.
- **Low risk:** All other patients are low risk cases and no adjuvant therapy is recommended.

1.2.2.11.1.5 Targeted therapy

They differ from chemotherapy drugs in the sense that they attack only cancer cells without causing damage to normal cells (Carrington 2015). Monoclonal antibody therapy is a type of targeted therapy that uses antibodies made in the laboratory from a single type of immune system cell. Bevacizumab is a monoclonal antibody that binds to a protein called vascular endothelial growth factor (VEGF) and can be used to treat advanced cervical cancer and recurrent cervical cancer.

Another targeted therapy Pazopanib which act as a kinase inhibitor and inhibits several kinase proteins (VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-A, PDGFR-B, FGFR-1, FGFR-3, Kit, Itk, Lck, and c-Fms) that promote growth and proliferation of tumor cells and also neo-angiogenesis. Pazopanib is showing encouraging results in treating cervical cancer (Monk, Willmott *et al*.).

1.2.2.11.2 Treatment according to stages

1.2.2.11.2.1 Stage 0 cancer

Carcinoma in situ (stage 0) requires local ablation or excision of the lesion and the transformation zone of the cervix. This can be done either by diathermy, laser, large loop excision of the transformation zone or knife cone biopsy depending on its size and location on the cervix (Jordan *et al*., 2008). Loop electrosurgical excision procedure (LEEP)/ laser therapy or conization/cryotherapy is recommended for ectocervical lesions. Conization which is also called cone biopsy is a procedure to remove a cone-shaped piece of tissue from the cervix and cervical canal. LEEP is a procedure that uses electrical current passed through a thin wire loop as a knife to remove abnormal tissue or cancer. Laser/cold-knife conization can be recommended for lesions involving endocervical canal in women desiring to preserve reproductive functions. Laser ablation a uses a laser beam as a knife to make bloodless cuts in tissue or to remove a surface lesion such as a tumor. In the post-reproductive age group stage 0 cervical cancer is treated by total abdominal hysterectomy.

1.2.2.11.2.2 Stage IA1 cancer

For Stage IA1 cancer is treated by surgery. Surgical options include total abdominal hysterectomy, radical hysterectomy, and conization. In total abdominal hysterectomy, the uterus along with ovarian tube and cervix are taken out through a large incision (cut) in the abdomen, while radical hysterectomy is the removal of the uterus, cervix, part of the vagina, and a wide area of ligaments and tissues around these organs. The ovaries, fallopian tubes, or nearby lymph nodes may also be removed. Lymph node dissection is required if the depth of invasions >3 mm with lymphovascular space invasion. For curative surgery, entire cancer must be removed with no cancer found at the margins of the removed tissue in per operative biopsy examination. This procedure is known as exenteration (Sardain, Lavoue et al. 2015).

1.2.2.11.2.3 Stage IA2

Radical hysterectomy (type II) with pelvic node dissection is recommended treatment for this stage (NCCN). Patients willing to restore reproductive function can undergo fertility-sparing surgery such as Trachelectomy, or complete removal of the cervix (Petignat and Roy 2007).

1.2.2.11.2.4 Stage IB, or IIA cancer

Stage IB1: Radical hysterectomy and bilateral pelvic lymphadenectomy with or without chemoradiotherapy.

Stage IB2 and IIA: The treatment options include radical radiation therapy (external plus intracavitary), radical hysterectomy (type iii) with bilateral pelvic lymphadenectomy and chemoradiotherapy.

1.2.2.11.2.5 Stage IIB, III A or IIIB cancer

Radiotherapy is the treatment of choice. Dramatic improvement in survival rate with chemoradiotherapy is observed in results from many large, randomized trials (Morris, Eifel et al. 1999). Hence, metastasized cancer such as stage IB2 and other advanced stages can be managed by Cisplatin based chemotherapy in combination with radiation (NCCN). On June 15, 2006, the US Food and Drug Administration approved the use of a combination of two chemotherapy drugs, hycamtin and cisplatin, for women with late-stage cervical cancer treatment (US FDA, 2006).

1.2.2.11.2.6 Stage IVA and IVB

For advanced disease palliative therapy is a mainstay. Radiation therapy is indicated for the control of bleeding and pain. Systemic chemotherapy is given for disseminated disease is recommended (NCCN).

1.2.2.12 Prognosis and survival rate

Cervical cancer prognosis depends on the stage of cancer. The chance of a survival is around 100% for women with cervical intraepithelial neoplasia (carcinoma in situ) (Encyclopedia of Women's Health). With standard treatment, 5 years survival rate, of women with stage I cancer and those with stage II cancer are 80 to 90% and 60 to 75% respectively. Survival rates decrease to 30 to 40% for women with stage III cancer and

15% or less of those with stage IV cancer 5 years after diagnosis (Beers 2005). The age at which cervical cancer is diagnosed will impact on the likely prognosis. Cancer Research UK shows that the younger a patient is, the better an outcome she can expect (Figure 1.13). Another study demonstrates that the 5-year survival rate of women under 65 (n = 221), between 65 and 74 (n = 56), and over 75 (n = 31) was 75%, 69%, 42%, respectively (P< 0.001) (Brun, Stoven-Camou et al. 2003).

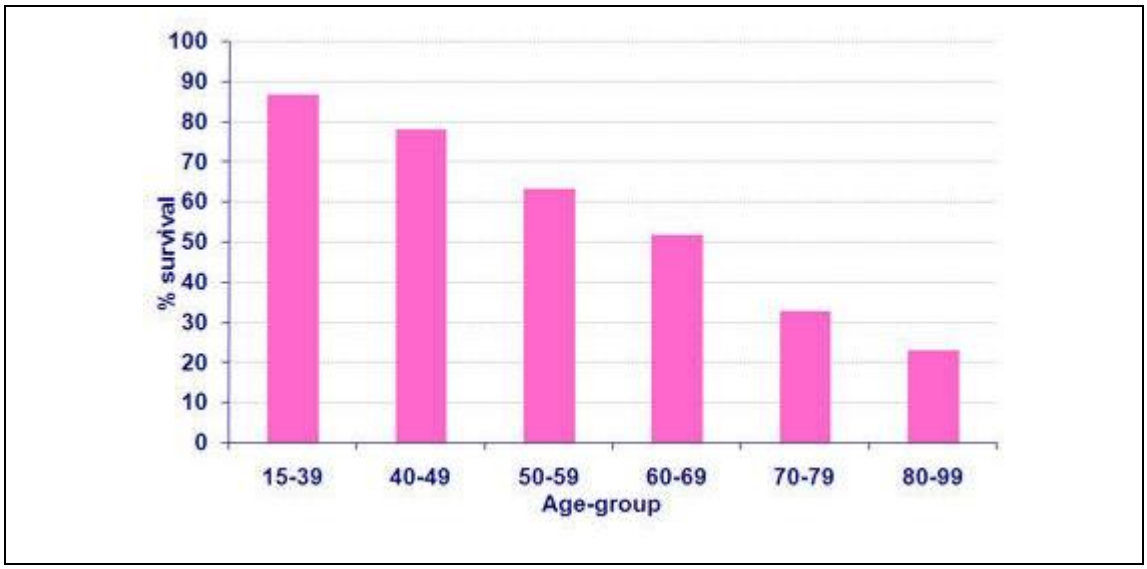

Figure 1.13: Cervical cancer survival rates by age. Source: (Cancer Research UK data).

1.2.2.13 Recurrence of cervical cancer

Recurrence rates according to FIGO staging are: stage IB- 10%, stage IIA - 17%, stage IIB- 23%, stages III and IVA - 42% and 74% respectively (Perez, Grigsby et al. 1995). Recurrence rate is 1.2% for tumor size less than 2 cm while for tumor size more than 2 cm, the rate is 21% (Estape and Angioli 1999). Cervix, uterus, vagina, parametria, bladder, ureters, rectum, and ovaries are most common sites of pelvic recurrence (Gadducci, Tana et al. 2010). Cervical cancer commonly metastasizes to distant sites such as paraaortic lymph nodes (81%), lungs (21%), and supraclavicular lymph nodes (7%) (Fagundes, Perez et al. 1992). Clinical features of recurrence depend on the site of metastasis and are characterized by weight loss, inferior limb edema, pelvic limb pain, vaginal bleeding, respiratory symptoms and supraclavicular lymph nodes swelling. Triad of weight loss, leg edema and pelvic pain is characteristic of recurrent disease (Friedlander and Grogan 2002). Treatment of recurrence is based on recurrence site (local, regional, and/ or distant), disease-free interval symptoms and the type of

primary therapy received. FDA approved Bevacizumab plus Cisplatin and Paclitaxel or Topotecan and Paclitaxel in 2014 for treatment of persistent, recurrent, or metastatic cervical cancer (Food and Administration 2016). A study describes the overall survival was 17 months with Bevacizumab and chemotherapy, whereas it was only 13.3 months with chemotherapy alone (Tewari, Sill et al. 2014).

1.2.2.14 Prevention of cervical cancer

1.2.2.14.1 Screening tests

Cervical cancer screening techniques used in different health care setup are VIA (visual inspection of the cervix), the Papanicolaou test or Pap smear, cervical cytology and HPV DNA testing. Visual inspection of the cervix as an alternative to the cytology-based detection tests is more appropriate to low-resource settings (Sankaranarayanan et al., 1998). Inspection is done by two methods, i.e., visual inspection with acetic acid (VIA) and visual inspection with Lugol's iodine (VILI). In the first method (VIA), which is also called as direct visual inspection (DVI), cervix is examined visually using a bright light after application of 3-5% acetic acid using cotton swab. The test is positive if an aceto-white area is observed near to squamocolumnar junction (SCJ). Acetic acid application may lead to reversible coagulation of intercellular proteins which also results in Aceto-whitening. However, the advantage of visual inspection approach is that it gives an immediate result for treatment. The Papanicolaou test or Pap smear which is widely used for the cervical cancer screening has decreased the prevalence and mortality of cervical tumor (Donta B et al, 2012). Cervical disease rate decreases up to 80% by screening with Pap test at regular intervals and routine follow up. Unusual results of Pap test suggest precancerous changes and guide the physician to carry out further investigation and start preventive treatment. Another potential screening technique is fluid based cytology that can detect HIV and Chlamydia which are also associated with cervical malignancy.

1.2.2.14.2 Human Papillomavirus Vaccines

Two different strategies may be employed for the development of vaccines against HPV based cancers, which are prophylactic and therapeutic vaccines.

1.2.2.14.2.1 Prophylactic Vaccines

Two prophylactic HPV vaccines named Gardasil® and Cervarix® are commercially available among which Gardasil is quadrivalent (HPV 6, 11, 16 and 18) and Cervarix is bivalent (HPV 16 and 18) (Harper et al., 2004). HPV major capsid protein, L1 is presented in the form of virus like particles (VLPs) in this prophylactic vaccines. This protein resembles virions, elicit virus-neutralizing antibodies and do not contain oncogenic viral DNA (Kirnbauer *et al.*, 1992). These two vaccines are recommended for vaccinating young adolescent girl at or before the onset of puberty. It is important for girls to get HPV vaccine before their first sexual contact - because they have not been exposed to HPV. Both of these vaccines are administered by intramuscular route in three doses (0, 1 or 2 and 6 months). Both vaccines have been shown to prevent potentially precancerous lesions of the cervix. They can prevent almost 100% of disease caused by the four types of HPV targeted by the vaccines (Garland, Hernandez-Avila et al. 2007).

1.2.2.14.2.2 Therapeutic vaccine

These VLP based HPV vaccines are obviously not therapeutic but the studies in animals and clinical trial data show the induction of a cell-mediated immune response to L1. It, therefore, requires further investigations to verify the ability of these vaccines in the regression of HPV infection or genital lesions. The therapeutic nature of Cervarix was examined in an interim analysis of a Costa Rican trial (Hildesheim *et al.*, 2007) and trial indicates that the vaccine did not induce the clearance of genital infections of other HPV types. In the Future II trial, the therapeutic activity of Gardasil was examined. No significant difference in the rate of CIN+ cases was observed in the vaccine vs. placebo control arm (11.1% vs. 11.9%) (Ault *et al*, 2007). These studies confirm that HPV VLPs based vaccines do not show therapeutic activity. The benefits of prophylactic vaccines in a broad public health perspective will be accomplished if these vaccines can reach to the women where the effective screening programs are absent.

1.2.2.15 Epidemiology

1.2.2.15.1 Worldwide prevalence

Globally, cervical cancer is the fourth most common cancer in women (World Cancer Report, 2014). The incidence of cervical cancer in developing countries has not reduced

significantly during the past three decades. Almost nine out of ten (87%) cervical cancer deaths occur in this regions, where it accounts for almost 12% of all female cancers. High prevalence of cervical cancer in the developing countries is associated with poor nutrition and general health status resulting in impaired immune function, ineffective screening programs and other social, behavioral, possibly genetic factors. In the developed world, by contrast, there has been a major decline in cervical-cancer mortality after the introduction of large-scale cytological testing such as Papanicolaou (Pap) smears. HPV screening by pap test in United States has resulted in a 70% reduction in the mortality from cervical cancer during the past 50 years (Bosch, Lorincz, Muñoz, Meijer, & Shah, 2002).

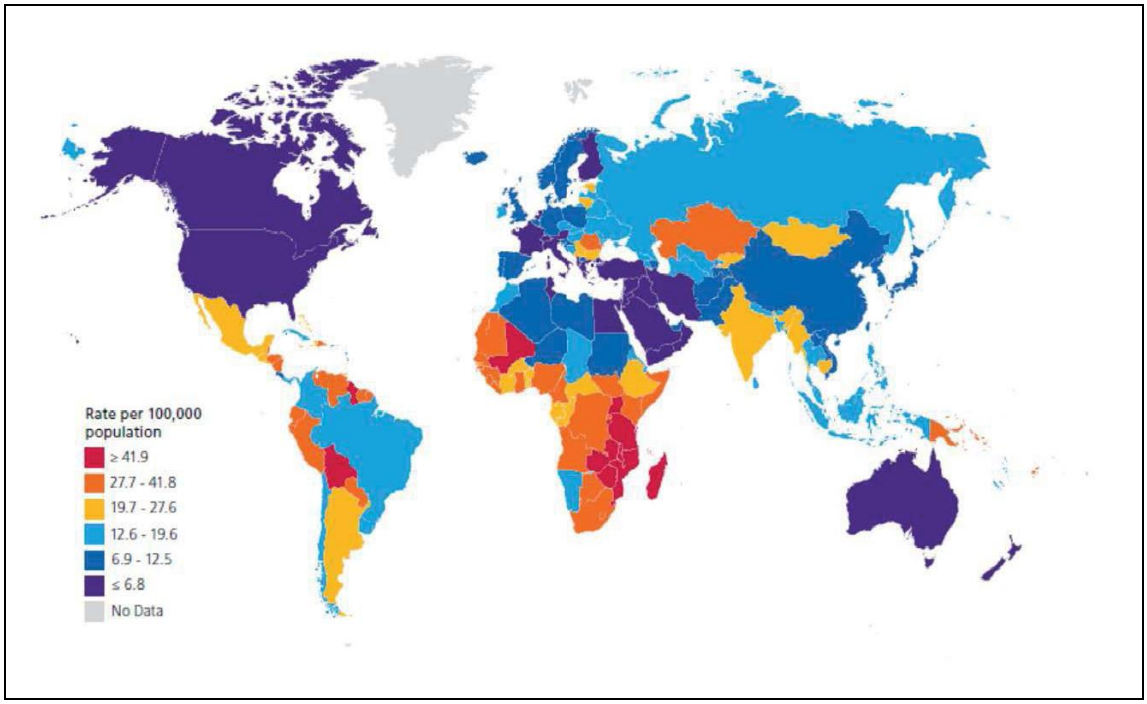

Figure 1.14: International Variation in Uterine Cervix Cancer Incidence Rates, 2012. Per 100,000, age standardized to the World Standard Population. Source: GLOBOCAN 2012.

1.2.2.15.2 Status in Bangladesh

Bangladesh has one of the highest levels of incidence and mortality rates due to cervical cancer among women. The prevalence of cervical cancer in Bangladeshi women is 25–30/100,000 (Khatun, Akram, Hussain *et al.* 2011). In a hospital based study of Bangladesh, 96.7% of 120 cervical cancer cases, 83.3% of 36 cases of CIN cases and 4.1% of 120 control women were HPV positive by hybridization (Sankaranarayanan, Bhatla *et al.* 2008). According to the Global Alliance for Vaccines and Immunization

(GAVI), Bangladesh stands 11th in the world in cervical cancer fatalities with 17.9 women dying in 100,000 due to the largely sexually transmitted killer disease every year (GAVI).

1.2.3 Genes with an effect on cancer progress

Cancer is a genetic disease - that is, cancer is caused by certain changes in genes that control the way our cells function, especially how they grow and divide. Cancer begins when genes in a cell become abnormal and the cell starts to grow and divide out of control.

Two of the main types of genes that play a role in cancer are oncogenes and tumor suppressor genes. An important difference between oncogenes and tumor suppressor genes is that oncogenes result from the activation of proto-oncogenes, but tumor suppressor genes cause cancer when they are inactivated.

1.2.4 Association of mutation in different cancer

Mutations happen often, and the human body is normally able to correct most of them. Depending on where in the gene the change occurs, a mutation may be beneficial, harmful, or make no difference at all. So, one mutation alone is unlikely to lead to cancer. Usually, it takes multiple mutations over a lifetime to cause cancer. This is why cancer occurs more often in older people who have had more opportunities for mutations to build up. But a mutation in the coding region or in the vicinity of either proto-oncogenes or tumor suppressor genes has a far more pronounced effect on cell cycle regulation and most often leads to cancer.

1.2.5 Gene mutation in cancer diagnosis and prognosis

Finding certain mutations in cells can confirm the diagnosis of that cancer. Testing cells for the mutation can also be used after diagnosis to see how the cancer is responding to treatment. Conversely, in some cancers, specific gene changes can be used to predict which patients are likely to have a better or worse outcome. This can help guide the intensity of treatment. Both of these are novel ideas which are not widely applied yet. But this line of thinking has a high promise and needs exploring.

Lately, mutation detection is being used for certain cancer types' diagnosis and prognosis. For example, the leukemia cells of patients with chronic myeloid leukemia (CML) contain a mutated gene called BCR-ABL. In order to be diagnosed with CML, this mutation must be present, so testing for this mutation is used to confirm the diagnosis. Conversely, patients with acute myeloid leukemia (AML) whose leukemia cells have a mutation in the FLT3 gene have a poorer prognosis than patients whose leukemia cells do not contain that mutation. Doctors may recommend more intense treatment, including stem cell transplant for someone whose leukemia cells have this mutation (ACS, 2016).

1.2.6 Specific genes of interest

Among a large number of genes involved in different cancer, some genes are commonly and frequently associated with the development of common cancers. The protein products of these genes control different signal transduction pathways and these pathways are involved directly or indirectly in various cell cycle and growth regulation mechanisms. Among them, *PIK3CA*, *EGFR*, and *KRAS* are of special interest in this research work.

1.2.6.1 *PIK3CA*

The human *PIK3CA* gene encodes the protein p110α which is a class I PI 3-kinase catalytic subunit. Phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) is composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The protein encoded by *PIK3CA* gene represents the catalytic subunit, which uses ATP to phosphorylate phosphatidylinositols (PtdIns), PtdIns4P and PtdIns P2.

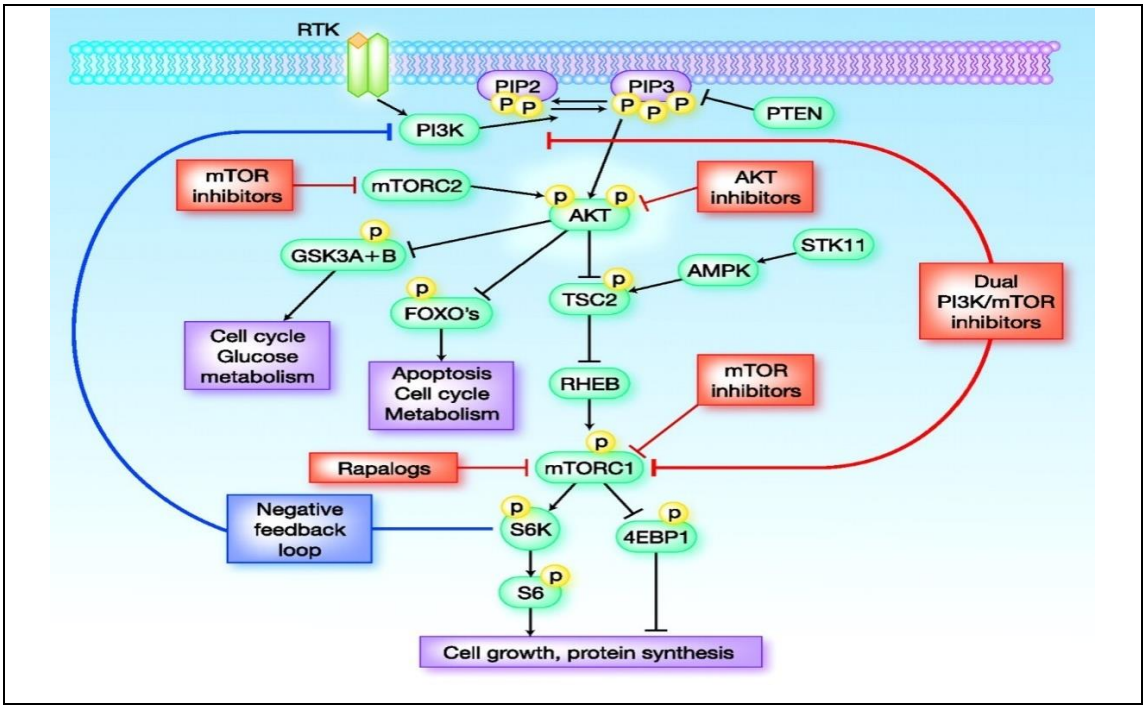

Figure 1.15: *PIK3CA* pathway. Source: (van der Heijden & Bernards, 2010).

1.2.6.1.1 Role in cancer

The involvement of p110α in human cancer has been hypothesized since 1995. Support for this hypothesis came from genetic and functional studies, including the discovery of common activating *PIK3CA* missense mutations in common human tumors (Samuels & Waldman, 2010). Common human tumors harbor activating *PIK3CA* missense mutations (Samuels *et al*., 2010). *PIK3CA* mutations are present in over one-third of breast cancers. Three hotspot mutation positions (GLU542, GLU545, and HIS1047) have been widely reported to date (Thirumal Kumar & George Priya Doss, 2017). *PIK3CA* mutation has been found to be oncogenic and is associated with cervical cancers (Ma et al., 2000).

1.2.6.2 *EGFR*

The *EGFR* gene provides instructions for making a receptor protein called the epidermal growth factor receptor, which spans the cell membrane so that one end of the protein remains inside the cell and the other end projects from the outer surface of the cell (Herbst, 2004). This positioning allows the receptor to attach (bind) to other proteins, called ligands, outside the cell and to receive signals that help the cell respond to its environment. Ligands and receptors fit together like keys into locks. Epidermal growth factor receptor binds to at least seven different ligands. The binding of a ligand to an epidermal growth factor receptor allows the receptor to attach to a nearby receptor protein (dimerize), turning on (activating) the receptor complex. These downstream signaling proteins initiate several signal transduction cascades, principally the MAPK, Akt, and JNK pathways, leading to DNA synthesis and cell proliferation (Oda, Matsuoka, Funahashi, & Kitano, 2005). As a result, signaling pathways within the cell are triggered that promote cell growth and division (proliferation) and cell survival.

EGFR gene encodes a protein called Epidermal Growth Factor Receptor (HER1 in humans) which is a transmembrane protein and a receptor for members of the epidermal growth factor family (EGF family) of extracellular protein ligands (Herbst, 2004). Upon activation by its growth factor ligands, *EGFR* undergoes a transition from an inactive monomeric form to an active homodimer (Yarden & Schlessinger, 1987). *EGFR* dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity. As a result, autophosphorylation of several tyrosine (Y) residues in the C-terminal domain of *EGFR* occurs (Downward *et al*., 1984). This autophosphorylation elicits downstream activation and signaling by several other proteins that associate with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains. These downstream signaling proteins initiate several signal transduction cascades, principally the MAPK, Akt, and JNK pathways, leading to DNA synthesis and cell proliferation (Oda et al., 2005). Such proteins modulate phenotypes such as cell migration, adhesion, and proliferation. Activation of the receptor is important for the innate immune response in human skin.

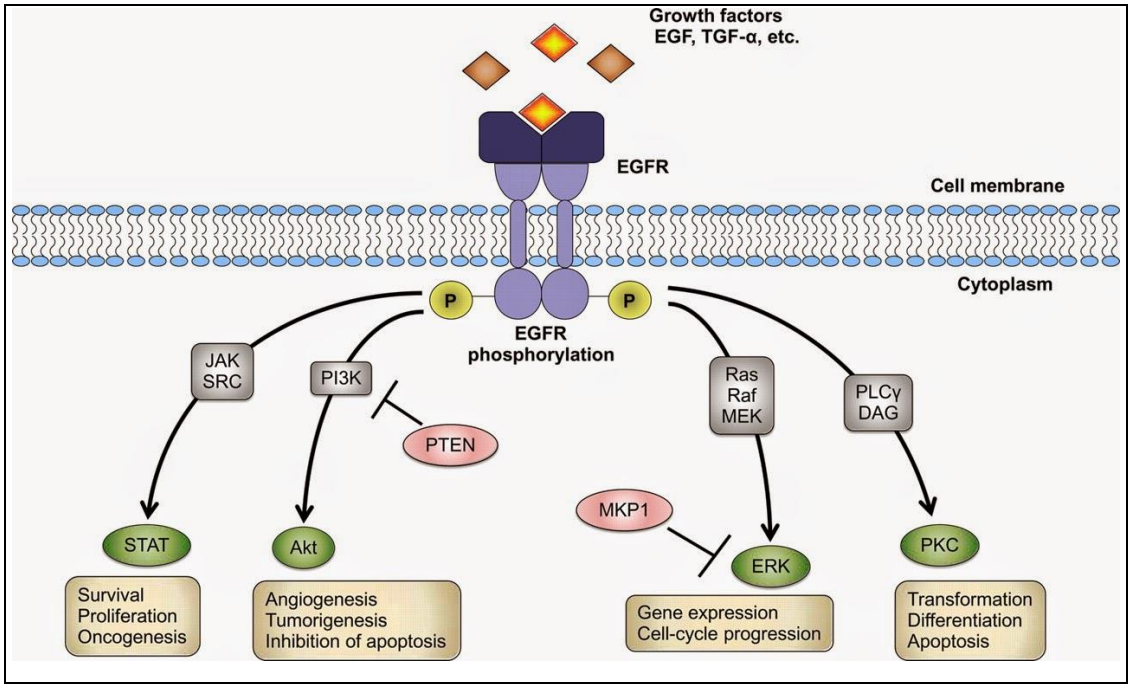

Figure 1.16: *EGFR* Pathway. Source: (Lee & Moon, 2011).

1.2.6.1.1 Role in cancer

Somatic mutations in *EGFR* gene results in constant activation of *EGFR* gene leading to uncontrolled cell division (Lynch *et al*., 2004). Mutations in *EGFR* gene are commonly found in a number of cancers, including squamous-cell carcinoma of the lung, anal cancers (Walker *et al*., 2009), glioblastoma and epithelial tumors of the head and neck (Kumar *et al*., 2013). Anticancer therapy directed against EGFR has been developed and called "EGFR inhibitors". They include gefitinib (Paez et al., 2004), erlotinib, afatinib, brigatinib, andicotinib. More recently AstraZeneca has developed Osimertinib, a third generation tyrosine kinase inhibitor (Greig, 2016). Additionally, imaging agents have been developed which identify EGFR-dependent cancers using labeled EGF or anti-EGFR (Lucas et al., 2015).

1.2.6.3 *KRAS*

The *KRAS* gene provides instructions for making a protein called K-Ras that is involved primarily in regulating cell division. As part of a signaling pathway known as the RAS/MAPK pathway, the protein relays signals from outside the cell to the cell's nucleus. These signals instruct the cell to grow and divide or to mature and take on specialized functions like - differentiation. The K-Ras protein is a GTPase, which means it converts a molecule called GTP into another molecule called GDP. The K-Ras protein acts like a switch, and it is turned on and off by the GTP and GDP molecules. To transmit signals, the K-Ras protein must be turned on by attaching to a molecule of GTP. The K-Ras protein is turned off when it converts the GTP to GDP. When the protein is bound to GDP, it does not relay signals to the cell's nucleus (NIH).

KRAS is a proto-oncogene corresponding to the oncogene first identified in Kirsten rat sarcoma virus (Tsuchida, Ohtsubo, & Ryder, 1982) and the gene product was first found as a p21 GTPase. *KRAS* acts as a molecular on/off switch, utilizing protein dynamics. Once it is allosterically activated, it recruits and activates proteins necessary for the propagation of growth factors, as well as other cell signaling receptors like c-Raf and PI 3-kinase.

1.2.6.3.1 Role in cancer

Somatic *KRAS* mutations are found at high rates in leukemia, colorectal cancer, pancreatic cancer (Almoguera et al., 1988) and lung cancer (San Tam et al., 2006). An activating mutation in the *KRAS* gene is responsible for various malignancies, including lung adenocarcinoma (Chiosea, Sherer, Jelic, & Dacic, 2011), mucinous adenoma, ductal carcinoma of the pancreas and colorectal cancer (Hartman, Davison, Foxwell, Nikiforova, & Chiosea, 2012). *KRAS* upregulates the GLUT1 glucose transporter, thereby contributing to the Warburg effect in cancer cells (Yun et al., 2009). However, the impact of *KRAS* mutations is heavily dependent on the order of mutations.

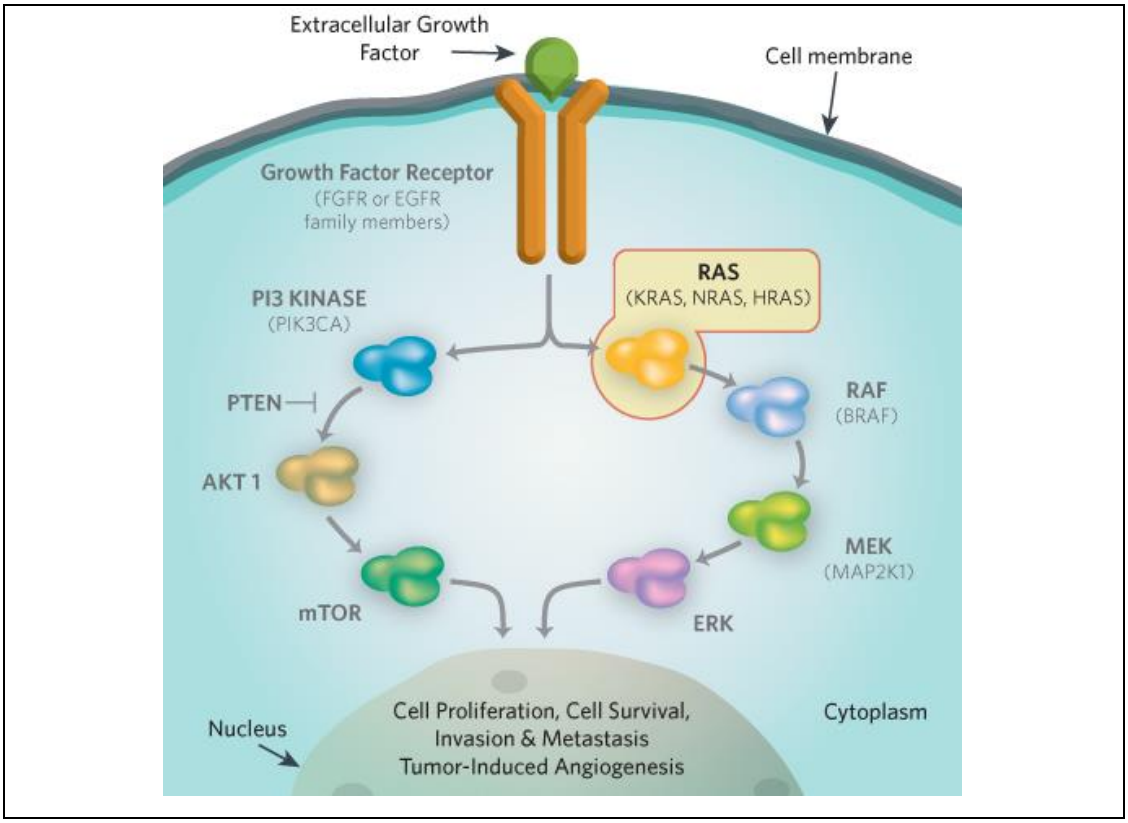

Figure 1.17: *KRAS* pathway. Source: (Center, 2013)

1.2.7 Relevance of these genes with cervical cancer

An oncogenic mutation which is not always induced by HPV infection affects regulation of cell cycle resulting in excessive growth or tumor of the cervix. Several studies have been done to determine whether specific genes have any significant mutations that can be attributed to cancer. Though research shows that variable oncogenic mutations in different genes rather than in specific one occur in cervical cancer, the PI3K/Akt/mTOR and MAPK/ERK pathways in cervical carcinoma are highly deregulated. A study shows the highest mutation rates were in the genes phosphatidylinositol 3-kinase, catalytic subunit α (*PIK3CA*) (31.3%); Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (8.8%); and *EGFR* (3.8%). *PIK3CA* mutation rates did not differ significantly between adenocarcinomas and squamous cell carcinomas (25% vs 37.5%, respectively; P = .33). In contrast, *KRAS* mutations were identified only in adenocarcinomas (17.5% vs 0%; P = .01), and a novel *EGFR* mutation was detected only in squamous cell carcinomas (0% vs 7.5%; P = .24 (Alexi A Wright et al., 2013). A study found that 14% of cervical cancer cases have *PIK3CA*

mutation (Miyake *et al*., 2008). Research suggests that HPV induce genomic instability leading to *EGFR* mutation (Kietpeerakool, Soonthornthum, & Srisomboon, 2013).

1.3 Rationale

In the context of low socioeconomic condition we are experiencing an increasing burden of cervical cancer disease and mortality rate is quite high. Studies related to statistics of cervical cancer (incidence, prevalence, death rate), cervical cancer screening test like visual inspection with acetic acid (VIA), PAP test, staging of cervical carcinoma, strains variant of HPV, HPV vaccination, treatment modalities have been carried out in Bangladesh. To best of our knowledge, any extensive research on mutation type, genomic location and mutation rates of cervical cancer in Bangladesh has not done yet. Without this information, decision of chemotherapy is most cases difficult and become non-specific treatment. Therefore, there is an opportunity for the molecular profiling of cervical carcinoma in our country.

1.4 Aims

Although early cervical cancer can be treated with surgery or radiation, metastatic cervical cancer is incurable and new therapeutic approaches are needed. The main goal of this study is to determine if any specific mutations in certain genes are associated with cervical cancer in Bangladeshi patients. The outcome of our result can help the clinicians in prevention and early diagnosis, understanding of prognosis and intervention in treatment modalities of this cancer.

1.5 Specific objectives

The specific objectives of this study are –

1) To identify and select mutation rich genomic ‘hotspot’ regions within genes that are most frequently mutated in cervical carcinoma,
2) To collect tissue samples of cervical carcinoma from Bangladeshi patients,
3) To amplify and sequence the selected genomic regions from DNA isolated from the collected samples,

4) To identify mutations and polymorphisms in selected regions from the sequence data,
5) To determine the frequency of mutations in the selected genes in sampled Bangladeshi patients,
6) To compare the determined mutation frequencies with known frequencies of other populations,
7) To determine the effect of the mutations on gene function and
8) To determine the effect of the exonic mutations on the structure and function of the respective proteins.

CHAPTER TWO

MATERIALS AND METHODS

This project aims at detection of specific gene mutation in Bangladeshi cervical cancer patients. This involves a series of distinct steps ranging from collection and processing of samples to *in silico* analyses of sequencing data.

Following is a summary chart showing the overall project workflow:

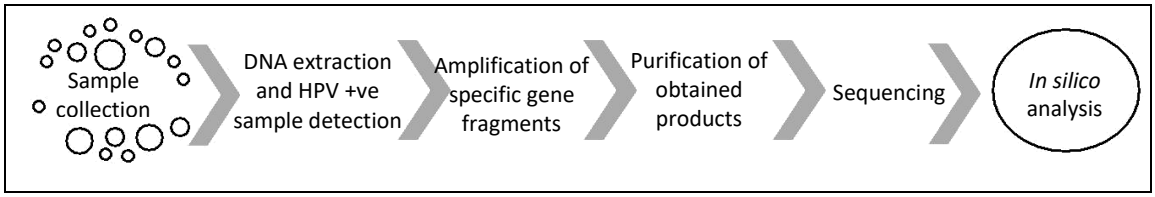

Figure 2.1: Summary of the project work.

Information related to chemicals, reagents, and apparatus used in this project can be found in Appendices I and II respectively.

2.1 Sample collection

2.1.1 Sources of samples

The cervical cancer mutation detection required cervical tissue specimens from women who developed cancer. Tissue samples were collected from cervical cancer patients from the National Institute of Cancer Research and Hospital (NICHR), Mohakhali, Dhaka and Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka. As per rule, all the specimens were collected by experienced personnel - either a trained nurse or a physician.

2.1.2 Ethical issues

2.1.2.1 Institutional clearance

All human tissue samples used in this study were used according to the National Health Research Strategy (NHRC) developed by National Research Ethics Committee (NREC) which is under Bangladesh Medical Research Council (BMRC). Institutional ethical clearance was taken from National Institute of Cancer Research and Hospital (NICHR), Mohakhali, Dhaka (Ref. no: NICRH/Ethics/2014/115). Institutional review board (IRB) clearance was taken from Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka (No.

BSMMU/2017/151). Ethical Clearance Committee of the University of Dhaka also approved all protocols (Ethical Clearance No. 30/Bio.Fac./2016-2017).

2.1.2.2 Patient consent

Before collection of the tissue sample, each patient was provided with a consent form (Appendix-III). Upon taking consent, information of the patient was written in a specific data collection sheet (Appendix-IV). All samples were collected with permission from the patients and the doctors involved. Patients were informed of all terms and conditions.

2.1.2.3 Maintenance of confidentiality

Every effort was made to keep patients' information records private. All personal medical information about the patients and any information obtained from the study of their specimen are preserved with security. Patient's name and identity was used only for data collection and not subject to be disclosed to a third party. All others, including employers, insurance companies, personal physicians, and relatives are refused/prohibited to access the information and to the samples, unless patient provides written permission, or unless we are required by law to do so. Anything that can identify patient is kept in private, protected files. An ID number was assigned to the patient, tissue samples, and information about his/her medical history. Only the investigator(s) named on this consent form was authorized to link the ID number to patient's name. Any research done on any of the samples was designed in a way that protects the privacy of the patients.

2.1.3 Collection procedure

2.1.3.1 Cervix visualization

During sample collection, the patient was kept in dorsal position. With appropriate illumination, Cusco's speculum was inserted through the vagina to visualize and fix the cervix. The cervix was inspected in this condition and relevant findings were noted.

2.1.3.2 Tissue specimen collection

The biopsy tissue specimen was extracted from cancer patients using a pair of sterile forceps, which was then kept in a screw-capped tube containing 2 mL PBS (Phosphate buffered saline). All tissue specimens were carefully labelled.

2.1.3.3 Transportation and storage

Samples were transported to the laboratory at the Department of Genetic Engineering And Biotechnology, University of Dhaka where all the necessary processing and analyses were carried out. The tissue samples were transported maintaining the cold chain in the form of an insulated ice box. After transport, tissue samples were stored at -20°C until further processing.

2.2 DNA extraction from samples

2.2.1 Tissue sample processing

Tissue sample processing and extraction of total DNA from the cervical sample were performed by using QIAamp® DNA Mini Kit (QIAGEN, Germany) according to the instructions provided in the user's manual.

The initial step was about extracting as much DNA possible through homogenization from the tissue sample. A small amount of tissue (<25 mg) was excised with a sterile scalpel and placed in a 1.5 mL centrifuge tube, which contained 180 µL Buffer ATL mixed with 20 µL proteinase K. The tissue was minced with a sterile micropestle to form a relatively even suspension. The tube was then sealed with paraffin film and incubated in a water bath at 56°C until the tissue was completely lysed. During the course of this incubation, the tube was taken out of the water bath and vortexed 2-3 times per hour. The centrifuge tube was centrifuged to remove drops from inside of the lid and the inner walls after the incubation. The incubation period was approximately 3-5 hours.

2.2.2 Tissue DNA extraction

Two hundred microliter Buffer AL (lysis buffer) was added to the sample and mixed by pulse-vortexing for 15 seconds to yield a homogenous solution. The lysing step has

a dual but interrelated purpose: to lyse the non-nucleic acid cell material on one hand and to retain as much nucleic acid as possible. Then the reaction tube was incubated at 70°C for ten minutes in a thermomixer (the shaking helps to dissolve any extant precipitate). Then the tube was briefly centrifuged to remove drops from the inside of the lid. Afterwards, 200µL ethanol (100%) was added to this and mixed again by pulse-vortexing for 15 seconds.

The next steps involved a thorough wash of the sample mixture to separate buffers and other materials from the desired nucleic acid. For this purpose, the mixture was applied to the QIAamp Spin Column placed in a 2 mL collection tube without getting the rim wet and was centrifuged at 6000 x g (8000 rpm) for one minute. After centrifugation, the QIAamp Spin Column was placed in a fresh 2 mL collection tube and the tube containing the filtrate was discarded. Five hundred microliter of Buffer AW1(Wash buffer 1) was applied to the QIAamp Spin Column and centrifuged at 6000 x g (8000 rpm) for one minute. Then the QIAamp Spin Column was placed in a fresh 2 mL collection tube and the tube containing the filtrate was discarded. After that, 500 µL of Buffer AW2 (Wash buffer 2) was applied to the QIAamp Spin Column and centrifuged at full speed (13,000 rpm) for three minutes. After discarding the filtrate containing tube, QIAamp Spin Column was placed in a clean 2 mL collection tube and centrifuged at 13,000 rpm for one minute to eliminate any chance of possible buffer AW2 carryover.

Lastly, for elution of DNA, the QIAamp Spin Column was placed in a fresh 1.5 mL microcentrifuge tube. Elution was done in two steps to ensure optimal DNA extraction from the column. Seventy microliter AE buffer was first added to the column and incubated for 5-10 minutes at room temperature, then centrifuged at 8000 rpm for at least one minute. The process was then repeated with an additional 50 µL AE buffer added to the column. The tube containing the extracted DNA was stored at -20°C for long-term storage.

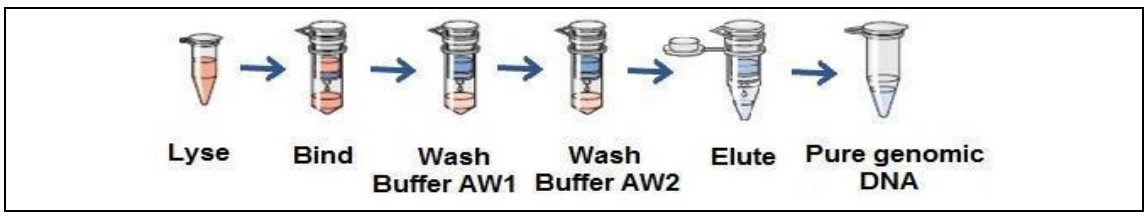

Figure 2.2: DNA extraction method using QIAamp® DNA Mini Kit (QIAGEN, Germany)

2.3 Specific gene amplification for mutation detection

For detection of gene mutation, specific genes were first chosen. These gene fragments were amplified using designed primer and by PCR reaction.

2.3.1 Selection of target gene

According to Wright et al. (Alexi A. Wright et al., 2013), the highest mutation rates in cervical cancer were in the genes phosphatidylinositol 3-kinase, catalytic subunit α (*PIK3CA*) (31.3%); then in Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (8.8%) and epithelial growth factor receptor (*EGFR*) (3.8%). Findings of Wright *et al.* reflects previous studies that shows high mutation rates in *PIK3CA* in cervical cancer. *KRAS* and *EGFR* mutations in cervical cancer are also reported in many studies. COSMIC (Catalogue of Somatic Mutation in Cancer) is an online database which reports somatic mutations in various cancers. In COSMIC, *PIK3CA*, *KRAS* and *EGFR* ranked top 3 among 20 genes that have high mutation rates in cervical cancer. Thus our target genes were selected.

2.3.2 Primer selection

Information about mutation hotspots of these genes in cervical cancer was gained from the COSMIC database. As 1 mutation cluster is found for *EGFR* gene, one set of primers was chosen for the gene. For *PIK3CA* and *KRAS* gene, 2 sets of primers were selected as 2 mutation hotspot were reported for these genes in COSMIC. So total 5 gene segments were amplified by 5 sets of primers. In this dissertation, the gene fragments are named as *PIK3CA*_1, *PIK3CA*_2, *EGFR*, *KRAS*_1, *KRAS*_2 and the primer sets are called as PIK3CA_1 primer, PIK3CA_2 primer, EGFR primer, KRAS_1 primer, KRAS_2 primer respectively. For primer designing, the full sequences of the three genes were downloaded from Ensemble database using BioMart portal. Keeping hotspot approximately in the middle position around 700bp were targeted to amplify for sequencing purpose. These sequences which represent specific gene fragment of above 3 genes were applied in primer 3 plus for primer designing. Among the different choices suggested by primer 3 plus, primers having best optimum parameters including primer Tm, GC%, CG Clamp, self-complementarity, 3' self-complementarity, 3' stability, repeat mispriming, pair repeat mispriming, template

mispriming, and pair template mispriming were selected. Location of selected primer set in the gene was determined and product size of final desired gene fragment to be amplified was matched with that of primer 3 plus. Table 2.1 presents the selected primers.

Table 2.1: Primers used for detection of gene mutation.

Target Gene		Primer Sequence	Product Size	GC%
***PIK3CA*_1**	F	AGACAATGAATTAAGGGAAAATGAC	690	32%
	R	GTTATACCACTCTTCATATAGCTCA		36%
***PIK3CA*_2**	F	AGTGGGGTAAAGGGAATCAAAAGA	533	41.7%
	R	GCAATTCCTATGCAATCGGTCT		45.5%
EGFR	F	ACGAAGCCTGTGTGTTTGGT	588	50%
	R	GGCAAAGGGAGTGGAAGGAA		55%
***KRAS*_1**	F	CTTAAGCGTCGATGGAGGAGT	545	52.4%
	R	ACCCTGACATACTCCCAAGGA		52.%
***KRAS*_2**	F	TGTCCGTCATCTTTGGAGCA	549	50%
	R	TTTCAATCCCAGCACCACCA		50%

(R= Reverse primer, F=Forward primer. Suffix with an underscore after the gene name indicates primer set number)

2.3.3 PCR amplification of desired gene fragments

2.3.3.1 Preparation of reaction mixture

Five different of PCR reactions were carried out for 5 primer sets.

Sterile 1.5 mL microcentrifuge tubes (Eppendorf, Germany) were taken and a master mix was prepared which included all the reagents for PCR reaction except the template DNA (Table 2.2). The amount of master mix was prepared according to the number of reaction. The master mix was aliquoted into PCR tubes before adding different extracted DNA samples. After adding template DNA to the reaction mixture, the PCR tube was capped and centrifuged briefly for mixing up the contents.

Table 2.2: Components of PCR reaction mixture

Reagents	Component volume in 5 µL reaction mixture	Final concentration
PCR grade water	16.375 µL	
10x Buffer	2.5 µL	1X
dNTPs (2.5 mM each)	2 µL	0.2 mM
Forward primer	1 µL	1 pmol/µL
Reverse primer	1 µL	1 pmol/µL
Taq Polymerase	0.125 µL	
Template DNA	2 µL	

2.3.3.2 PCR reaction condition

PCR tubes containing master mix and template DNA were placed in thermal cycler (BioRad, USA). The PCR reaction was performed according to the set program (Table 2.3) using thermal cycler.

Table 2.3: Condition for PCR reaction in thermal cycler.

Steps	Cycle number	Temperature	Duration	Events
Step 1	1	95°C	10 minutes	Initial denaturation
Step 2	35	94°C	45 seconds	Denaturation
		Different for different primers.	1 minutes	Annealing
		72°C	45 seconds	Elongation
Step 3	1	72°C	7 minutes	Final extension
Step 4	1	4°C	∞	Final hold

We optimized PCR reaction condition of different primers we used. Using prediction of annealing temperature by primer 3plus tool and given melting point by the suppliers, we performed several trials (gradient PCR) to find optimum condition and finally and fixed annealing temperature (Table 2.4) for the primers that gave us best agarose gel electrophoresis result.

Table 2.4: Annealing temperatures for 5 different primer sets

Primer used	Annealing temperature for PCR (°C)
PIK3CA_1	**57**
PIK3CA_2	**64**
EGFR	**63**
KRAS_1	**62**
KRAS_2	**65**

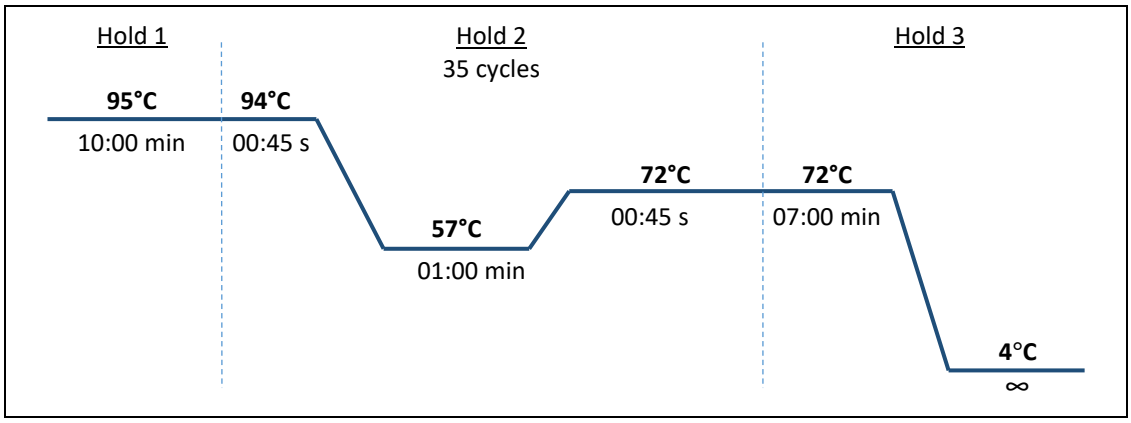

Figure 2.3: Graphical representation of PCR condition for PIK3CA_1 primer set

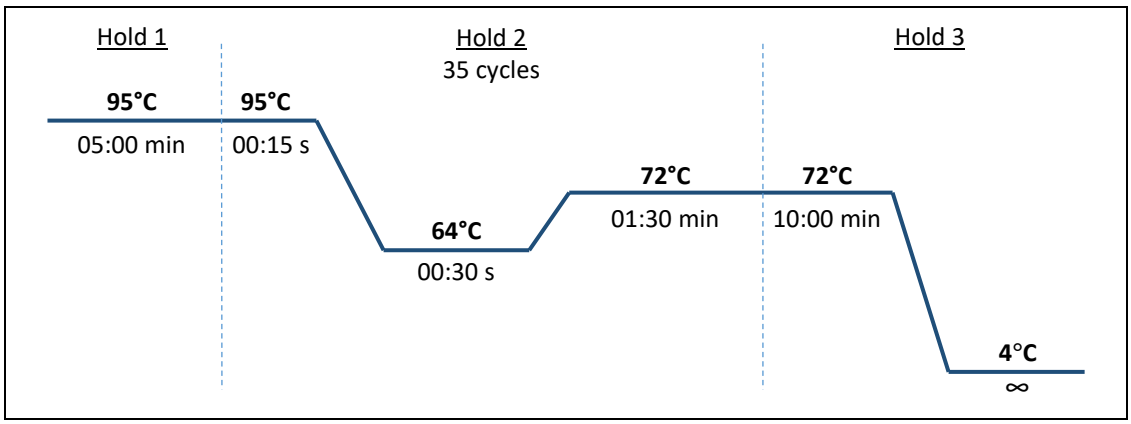

Figure 2.4: Graphical representation of PCR condition for PIK3CA_2 primer set

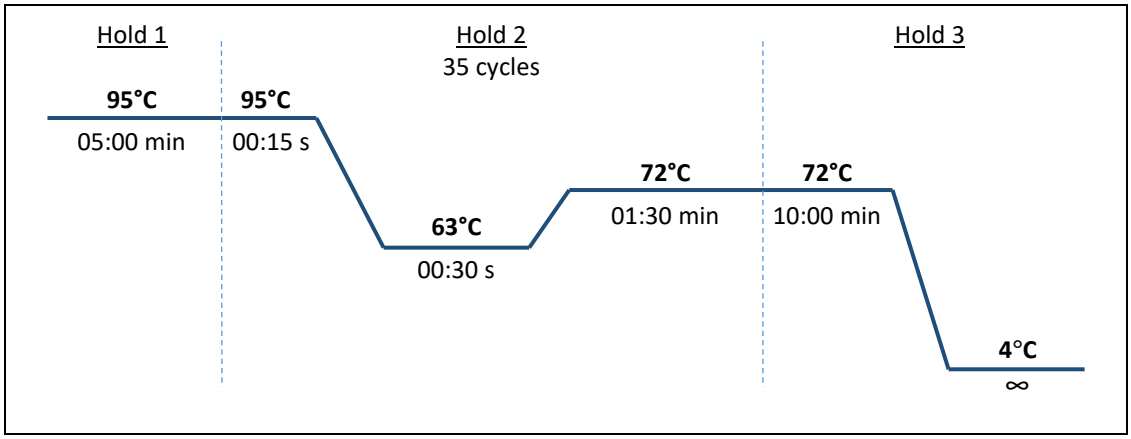

Figure 2.5: Graphical representation of PCR condition for EGFR primer set

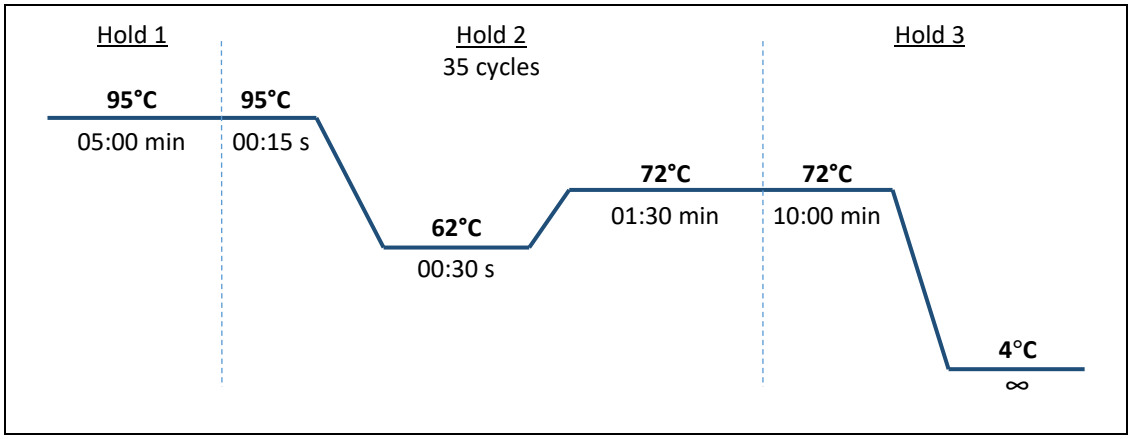

Figure 2.6: Graphical representation of PCR condition for KRAS_1 primer set

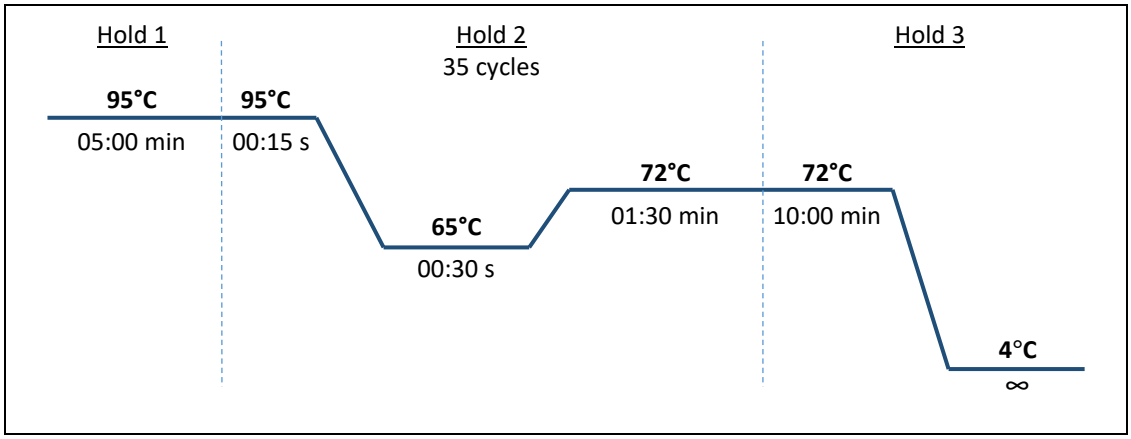

Figure 2.7: Graphical representation of PCR condition for KRAS_2 primer set

2.4 Post-PCR detection of amplified DNA by electrophoretic analysis

The successful amplification of the desired regions was examined by agarose gel electrophoresis method. The PCR products were resolved in 1.5% agarose gel to analyze the specific band of the amplicon.

2.4.1 Preparation of agarose gel

1.5% agarose gel was prepared by dissolving agarose (Sigma, USA) in 1X Tris-borate EDTA (TBE) buffer (Appendix I) to give a final concentration of 1.5% agarose and was heated in a microwave oven for about 2-2.5 minutes. The melted agarose was allowed to cool to about 50°C. Two microlites of Ethidium Bromide (EtBr) (concentration 0.5 µg/mL) was added in the melted agarose and mixed completely by gentle agitation. The melted agarose with EtBr was poured into gel electrophoresis unit (Sigma, USA) with spacers and comb to form wells. After solidification of the gel, the comb was removed. Then the gel was submerged in 1X TBE buffer in a gel electrophoresis unit.

Five microliters of each PCR product was mixed with 1 µL of 6X gel loading dye (Appendix). The mixture was slowly loaded into the well using disposable micropipette tips. Marker DNA of known size (100bp ladder) (Bioneer, South Korea) was loaded in one well to determine the size of the PCR products. Electrophoresis was carried out at 95 volts for approximately 45 minutes.

2.4.2 Visualization of the gel

The DNA bands intercalated with EtBr were observed on a UV transilluminator (VilberLourmat, France). Photographs were taken using a gel documentation system (VilberLourmat, France) and bands were analyzed.

2.5 PCR product purification for sequencing

For sequencing, first PCR reactions were carried out to obtain 50 µL products. The samples which showed positive results for the previous PCR reactions were used as DNA templates. Five microliter product was used for gel electrophoresis to make sure if the product of right size was present. These products needed to be cleaned up for

efficient sequencing since it is imperative to make sure only the amplicon DNA remains in the solution, with the exclusion of the polymerase, primers, or individual nucleotides. For this purpose, PureLink PCR Purification Kit (Invitrogen, USA) was used. The protocol was adopted from the manual supplied with the kit with minor modifications.

First, in a sterile 1.5 mL microcentrifuge tube, 45 µL of PCR product was mixed with 180 µL B3 (Binding buffer). The kit includes two binding buffer solutions- one of them, B3, is specifically used to preclude elution of DNA less than 300 bp in length. Using this makes sure that no small fragment of non-specific DNA product remains in the sample.

The PCR product-buffer mixture was then loaded with a micropipette into the supplied PureLink PCR Spin Column fitted in a collection tube, which was then centrifuged at 11,000 g (14,000 rpm) for one minute. The filtrate was discarded from the collecting tube and the PureLink PCR Spin Column was fitted in it again.

In the washing step, 650µL of the supplied wash buffer was added to the column. The spin column, fitted into the collection tube, was centrifuged at 11,000 g (14,000 rpm) for 1 minute, and then once more after discarding the filtrate at maximum speed (13,000 rpm) for 4 minutes.

Finally, in the elution step, the spin column was placed in a supplied elution tube. Fifty microliter elution buffer was applied to the column, which was then left at room temperature for 5-10 minutes. Afterwards, the column fitted in the elution tube was centrifuged at 13,000 rpm for 2 minutes, which resulted in a purified DNA solution.

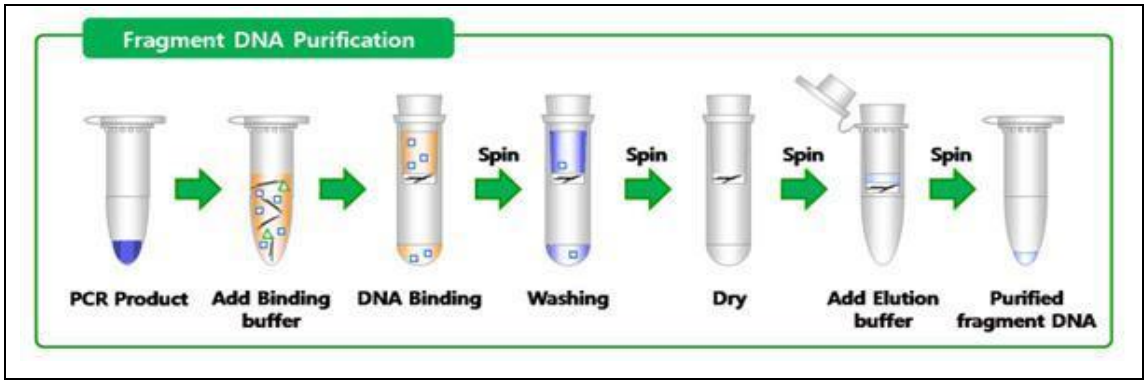

Figure 2.8: Purification of PCR products

Purified PCR products were measured as ng/µL using Nanodrop™ spectrophotometer (Thermo Scientific, USA). The ratio between the readings at 260 nm and 280 nm (OD 260 /0D 280) provides an estimate of the purity of the DNA. Pure DNA preparations have OD 260/OD 280 values of 1.8. The tubes containing the purified DNA were then stored at -20°C until sequencing.

2.6 Sequencing reaction

The purified DNA fragments were sequenced by a cycle sequencing strategy, using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Since fragments were longer than 500 bp, two primers were used to sequence the fragment from both sides.

The protocol adopted was based on the manual supplied with the kit. In this section, the cycle sequencing reaction and clean-up procedures are briefly discussed. After the reaction is run, the products are purified, followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems, USA).

The basic sequencing protocol is illustrated below:

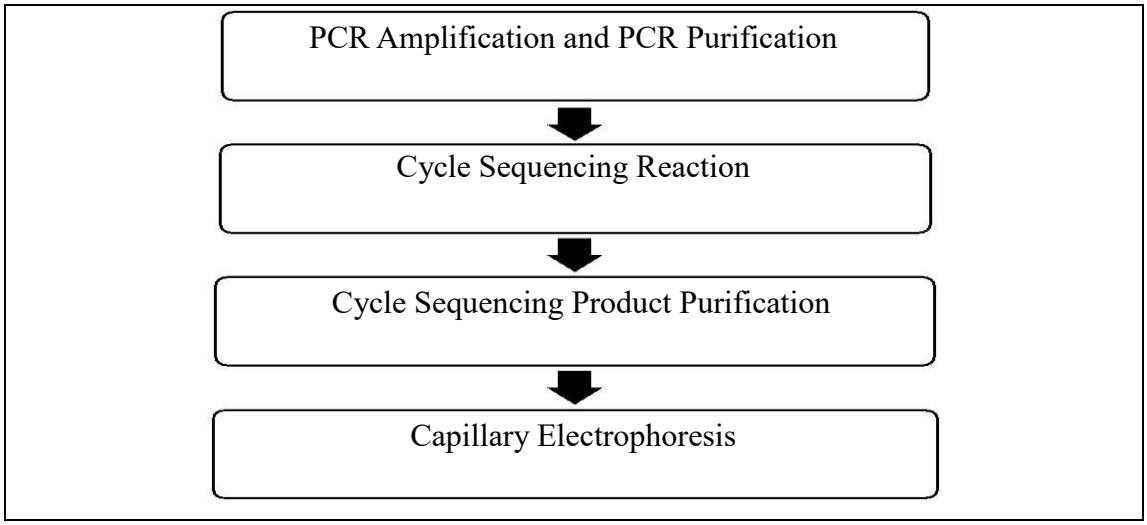

Figure 2.9: Flowchart of basic sequencing protocol

The reaction mixture of cycle sequencing was prepared for either 96-well reaction plates or microcentrifuge tubes to perform cycle sequencing of purified PCR products. The reaction mixture was prepared as in Table 2.5.

Table 2.5: Preparation of Reaction mixture

Reagent	Quantity
Terminator Ready Reaction Mix	8.0 µL
Template	variable
Primer	3.2pmol
Deionized water	Up to 20µL
Total Volume	**20µL**

The reaction mixture was added to each tube and mixed well followed by spinning the tubes briefly. The BigDye® Terminator 3.1 Sequencing Buffer (5X) is supplied at a 5X concentration. The amount of 5X sequencing buffer to the sequencing reaction is dependent on the stage at which someone wants to terminate the reaction. For a half reaction in 20 µL final volume, the sequencing buffer was used according to the Table 2.6.

Table 2.6: Reaction mixture for sequencing using BigDye® Terminator v3.1 Sequencing Buffer

Reagent	Concentration	Quantity
Ready Reaction PreMix	2.5X	4.0 µL
BigDye Sequencing Buffer	5X	2.0µL
Template	-	variable
Primer	-	3.2pmol
Deionized water	-	Up to 20µL
Total Volume	1X	**20µL**

Tubes were placed in a thermal cycler and the volume was set. The cycle sequencing started with initial denaturation at 96º C for one minute followed by 25 steps of 96º C for 10 seconds, 50º C for 10 seconds and 60º C for 4 minutes. The reactions were kept in hold at 4º C until the start of purification step of the extension products. The product was then spin down in a microcentrifuge. The extension products were purified by

Ethanol/EDTA precipitation method. Before starting the precipitation method, reaction plate was removed from thermal cycler and spun briefly. Then 5 µL EDTA followed by 60 µL of 100 % ethanol was added to each well. The reaction plate was sealed with aluminium tape and mixed by inverting 4 times. After that, reaction plate was incubated at room temperature for 15 minutes. After spinning the plate up to 185 ×g, 60 µL of ethanol was added to each well. The centrifuge machine was set at 4º C and spun at 1600 ×g for 15 minutes. In last step, the plate was inverted again and spun at 185 ×g for exactly one minute. The plate was then removed from the centrifuge and sealed with aluminium tape at 4°C. The sample was then analyzed by ABI Genetic Analyzer (Applied Biosystems®, USA).

2.7 *In silico* analysis

In silico analysis was performed in the following phases:

- Analysis in search of mutations in the target genes
- Genomic and statistical analysis of the identified mutations
- Analysis of the consequences of mutation on protein structure and function

2.7.1 Analysis in search of mutations in the target genes

After gene fragment sequences were obtained, they were carefully analyzed in order to detect possible mutation. For this reason, sequence of each strand was separately compared to the NCBI GenBank (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2005) database using the NCBI BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990). If any inconsistency was found after the alignment, the corresponding chromatogram was scrutinized to determine whether the discrepancy was due to an error in sequencing or by a valid change of base in the sequence. If a valid mutation was found, it was recorded. Mutation location was determined by referring to the GenBank database containing information about the genes of interest. Additionally, types of the found mutations were categorized as well.

2.7.2 Genomic and statistical analysis of the identified mutations

2.7.2.1 Identification of effect of exonic and intronic mutation on gene function

Ensembl Variant Effect Predictor (VEP) (McLaren et al., 2016) tool was applied in search of the effects of the variants of the mutations across Ensembl and RefSeq transcripts databases using a BED file containing the mutation information**.** This tool outputs the mutation affected genes and transcripts, mutation location in the gene, functional consequence of the mutation and also use of different other integrated tools which include LoFtool, SIFT, Polyphen-2 and mutation assessor.

2.7.2.2 Prediction of the functional intolerance of genes due to mutation

LoFtool (Fadista, Oskolkov, Hansson, & Groop, 2016) provide a rank of genetic functional intolerance and consequently susceptibility to disease due to the loss of function (LoF) mutation. LoFtool was used in order to find out the intolerance score based on loss-of-function of variants of the mutation. The Smaller the LoF score, the chances are more to become intolerant.

2.7.2.3 Determination of the frequency of exonic mutation

Mutations that were found in the exons were determined. Every particular exonic mutation frequency of was also calculated to find out among these three genes which one contains the most exonic mutations in this study.

2.7.3 Analysis of the consequences of mutation on protein structure and function

2.7.3.1 Prediction the effect of non-synonymous mutation on protein function

In order to find out the effects of missense mutations on the protein level, two different scores, SIFT and Polyphen scores, were estimated from the integrated calculation of the VEP tool.

SIFT (Kumar, Henikoff, & Ng, 2009) predicts whether an amino acid substitution due to a single nucleotide variation affects the protein function. The higher the SIFT score, the higher chances of being tolerated in the protein structure and function changes. Similarly, PolyPhen-2 (Adzhubei et al., 2010) tool predicts the possible impact of an amino acid substitution on the structure and function of a human protein. But in this case, lower the Polyphen score, smaller the chance of mutation to have a deleterious effect on the protein function and structure.

MuPro tool (Cheng, Randall, & Baldi, 2006) was also used which uses sequence and structure related information in order to predict the effect of a mutation on the stability and function of the target protein.

2.7.3.2 Determination of the location of mutant amino acid in the protein

In order to find out in which functional domain of the protein a particular mutant amino acid is located, the MutationMapper tool (Vohra & Biggin, 2013) was used and the missense mutations were mapped and function of the particular domain was searched.

2.7.3.3 Homology modeling and structure comparison between the wild-type and mutant protein

After mutation detection, amino acid sequences corresponding to the nucleotide sequences was carried out to check whether any of the found mutations resulted in a change of amino acid. Amino acid change may lead to protein structure change, so mutant protein was modeled against the wild-type one using the SWISS-MODEL tool (Biasini et al., 2014). The generated models have been observed using the UCSF Chimera tool (Pettersen et al., 2004) and the models of the mutant and normal proteins were matched to find out the location of the target site of the amino acid and its functional domain.

CHAPTER THREE

RESULTS

Worldwide cervical cancer remains one of the leading causes of cancer-related deaths (The Cancer Genome Atlas Research, 2017). With the advancement of the early detection and treatment of pre-invasive disease, invasive cervical cancer rates have steadily decreased over the past several decades. However, treatment of advanced or recurrent cervical cancer is still limited, resulting in poor survival (Kitagawa et al., 2015). Although early cervical cancer can be treated with surgery or radiation, new therapeutic approaches are needed for metastatic cervical cancer (Uyar & Rader, 2014). Oncogenic mutations in human genes which are not induced by HPV infection play a major role in the formation and progression of cervical cancer. Whereas some researches on cervical carcinoma have been carried out in Bangladesh which are on epidemiology, screening tests, staging of cervical carcinoma, HPV vaccination etc.; research related to genetic marker or gene mutation in cervical cancer has not been done yet in Bangladesh. In this study, genetic mutations in specific host genes were detected.

3.1 Molecular detection of specific gene fragments in cervical cancer samples by Polymerase Chain Reaction (PCR)

Three specific genes were chosen to be amplified for this study. *EGFR* gene fragments were amplified using one set of primers for each gene. Since *PIK3CA* and *KRAS* genes were indicated to harbor more mutation hotspot than *EGFR*, two sets of primers were used to amplify two different regions of the genes. In total, forty-six extracted DNA samples were used in this study.

3.1.1 Amplification of the *PIK3CA* gene fragment

3.1.1.1 Amplification of *PIK3CA* gene using PIK3CA_1 primer pair

Using the PIK3CA_1 primer set, the particular gene fragment was amplified for the 46 samples. PCR products were estimated to be sized approximately 700 bp on agarose gel electrophoresis (Figure 3.1).

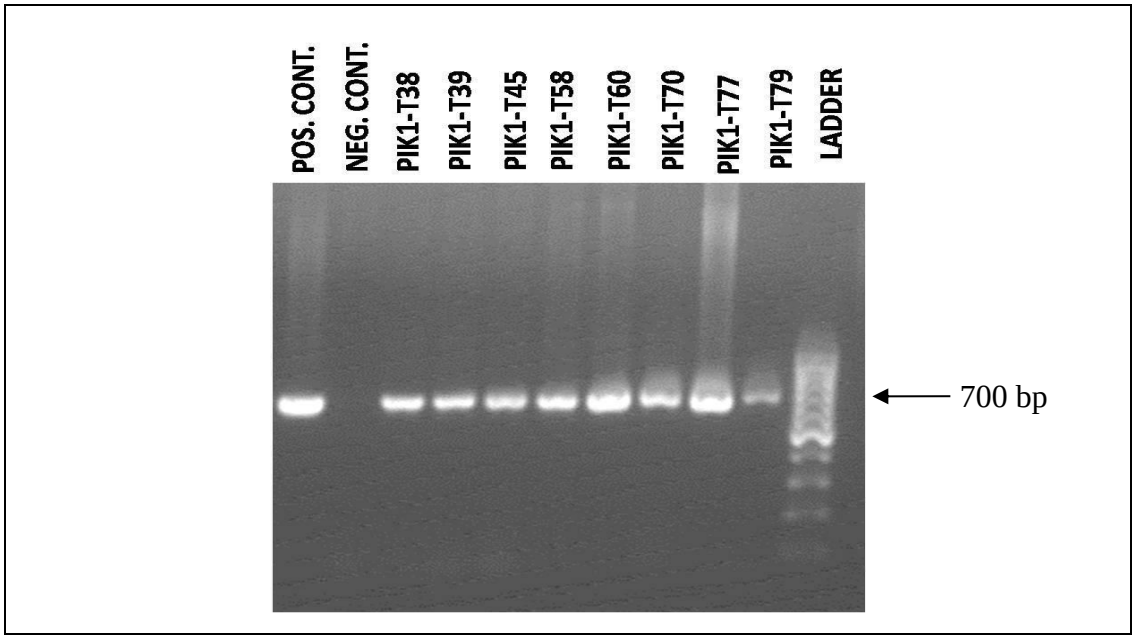

Figure 3.1: Agarose gel electrophoresis of PCR specific amplicon (~700bp) using PIK3CA_1 primer pair. Lane 1 = positive control, Lane 2 = Negative control, Lane 3-10 = samples, Lane 11 = 50bp ladder (Bioneer, USA).

3.1.1.2 Amplification of *PIK3CA* gene using PIK3CA_2 primer pair

Using the PIK3CA_2 primer set, 46 samples were amplified. PCR products showed band on agarose gel electrophoresis at approximately 600bp (**Figure** 3.2).

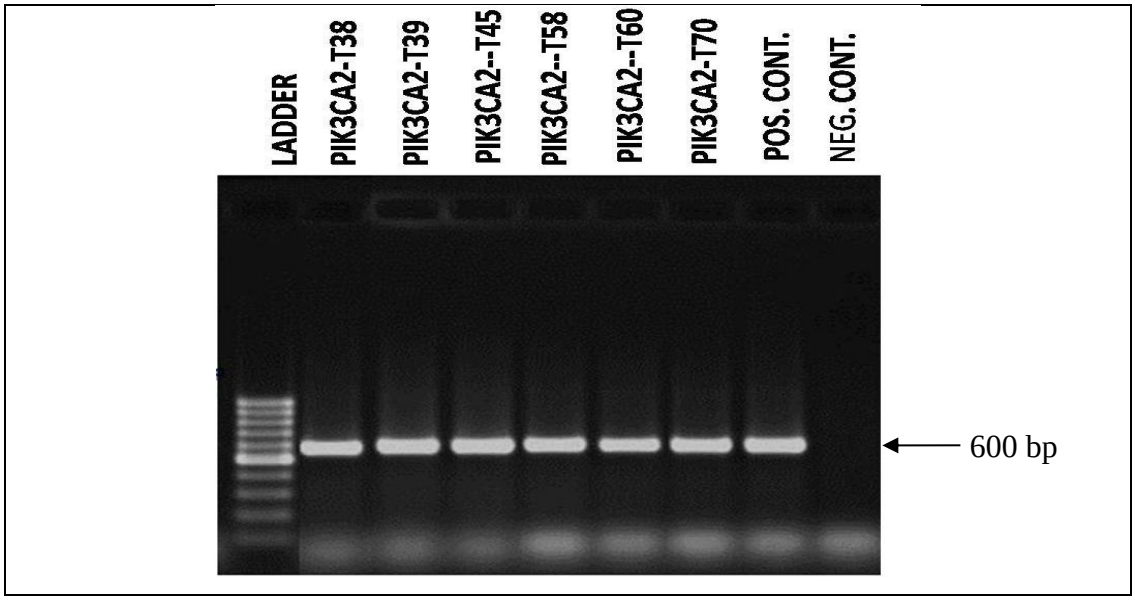

Figure 3.2: Agarose gel electrophoresis of PCR specific amplicon (~600 bp) using PIK3CA_2 primer pair. Lane 1 = 100 bp ladder (Bioneer, USA), Lane 2-7 = samples, Lane 8= positive control Lane 9 = Negative control.

3.1.2 Amplification of *EGFR* gene

Using the *EGFR* primer set, DNA samples from 46 patients were amplified. PCR products showed band on agarose gel electrophoresis at approximately 600 bp (Figure 3.3).

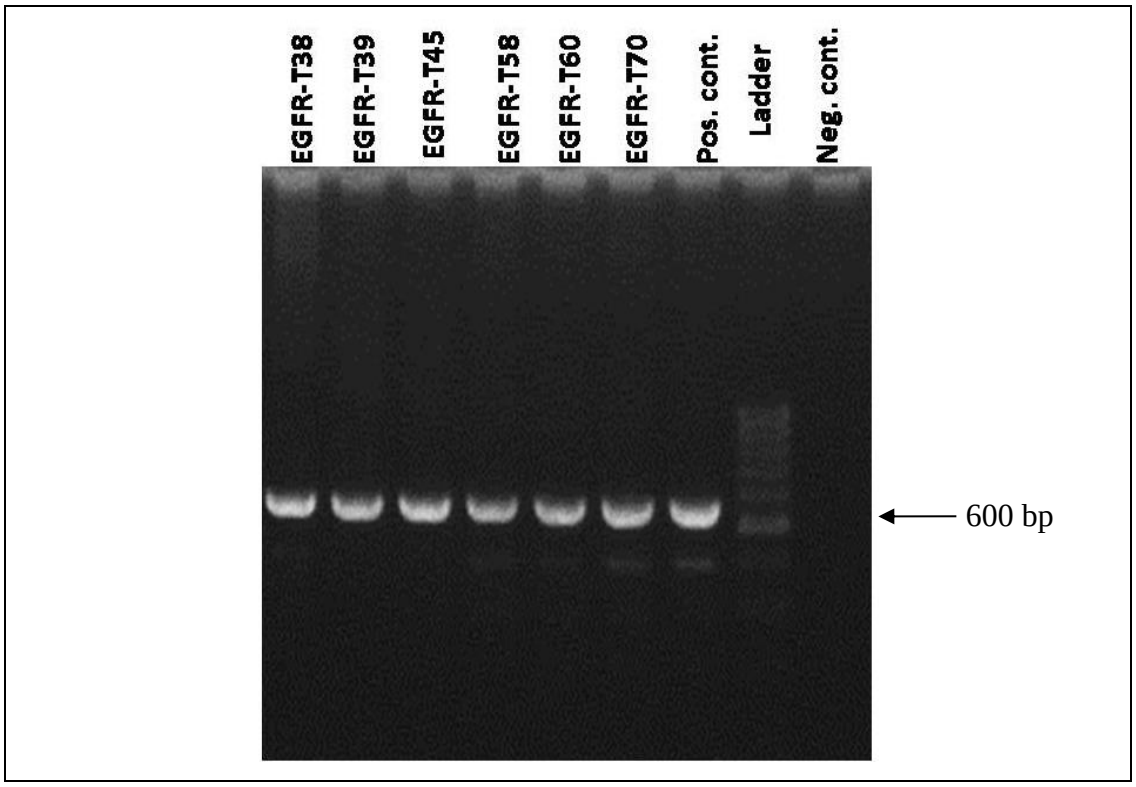

Figure 3.3: Agarose gel electrophoresis of PCR specific amplicon (600 bp) using *EGFR* primer pair. Lane 1-6 = samples, Lane 7 = Positive control, Lane 8 = 50 bp ladder (Bioneer, USA), Lane 9 = Negative control.

3.1.3 Amplification of *KRAS* gene

3.1.3.1 Amplification of *KRAS* gene using KRAS_1 primer pair

Using the KRAS_1 primer set, extracted DNA samples were amplified. PCR products showed band on agarose gel electrophoresis at approximately 550bp (Figure 3.4).

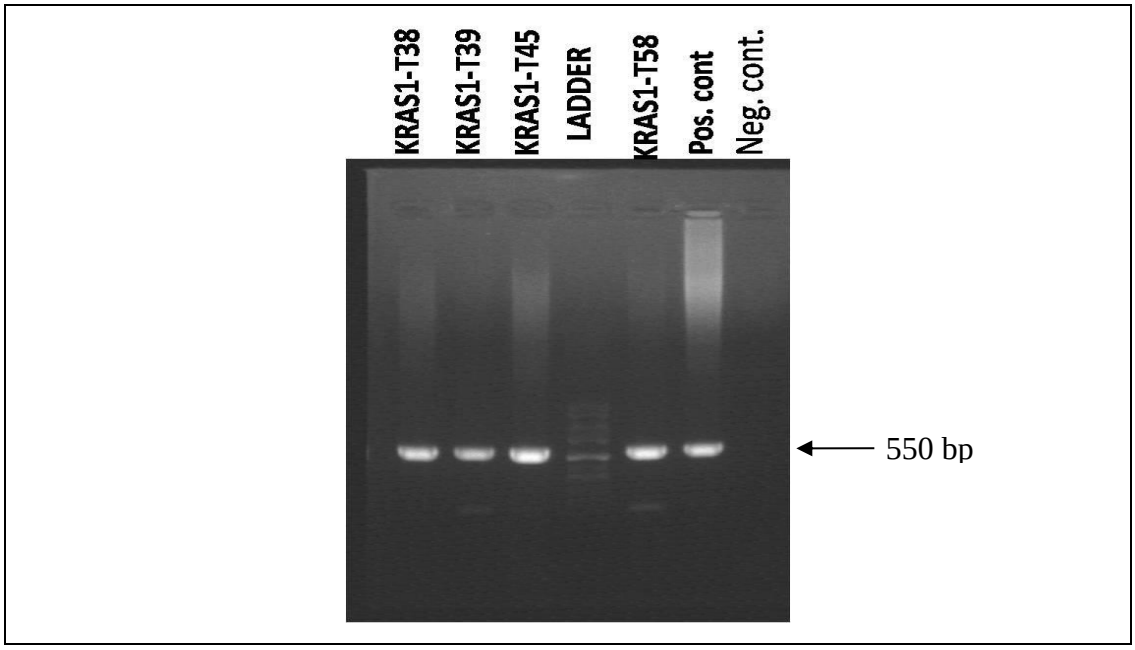

Figure 3.4: Agarose gel electrophoresis of PCR specific amplicon (~550bp) using KRAS_1 primer pair. Lane 1-3 = sample, Lane 4 =50 bp ladder (Bioneer, USA) Lane 5 = sample, Lane 6 = Positive control, Lane 7 = Negative control.

3.1.3.2 Amplification of *KRAS* gene using KRAS_2 primer pair

Using the KRAS_2 primer set, 46 samples were amplified. PCR products showed band on Agarose gel electrophoresis at approximately 550bp (Figure 3.5).

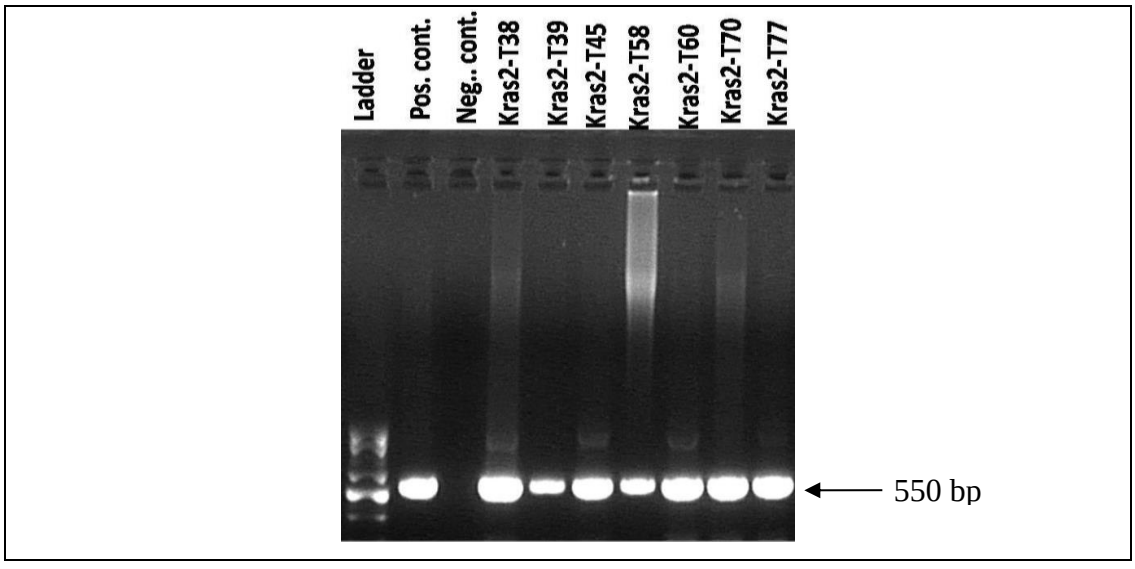

Figure 3.5: Agarose gel electrophoresis of PCR specific amplicon (~550bp) using KRAS_2 primer pair. Lane 1 = 50 bp ladder (Bioneer, USA), Lane 2 = positive control, Lane 3 = Negative control, Lane 4-10 = samples.

3.2 Sequence analysis

After sequencing was carried out using purified PCR products and chromatogram quality checked and manual editing, clean sequence data analysis was done in three steps. They are:

- Matching with database
- Detection of change in base
- Checking with chromatogram to ensure the validity

All the samples were sequenced using both forward and reverse primers and then compared with each other to ensure correct sequencing.

3.2.1 Sequence data analysis by matching with database

In total, 230 gene fragments were sequenced (both strands). After obtaining sequence data, they were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool (BLAST). All the sequences were matched with *Homo sapiens* chromosome primary assembly. *EGFR* gene is located on chromosome 7, *KRAS* gene is located on chromosome 12 and *PIK3CA* gene is located on chromosome 3.

3.2.2 Detection of mismatched base

All the BLAST results were carefully scrutinized to detect any mismatched base. Following is an example of a base mismatch found through BLAST analysis. There were two BLAST results, one showing no mutation (Figure 3.6) and the other (Figure 3.7) showing mutation.

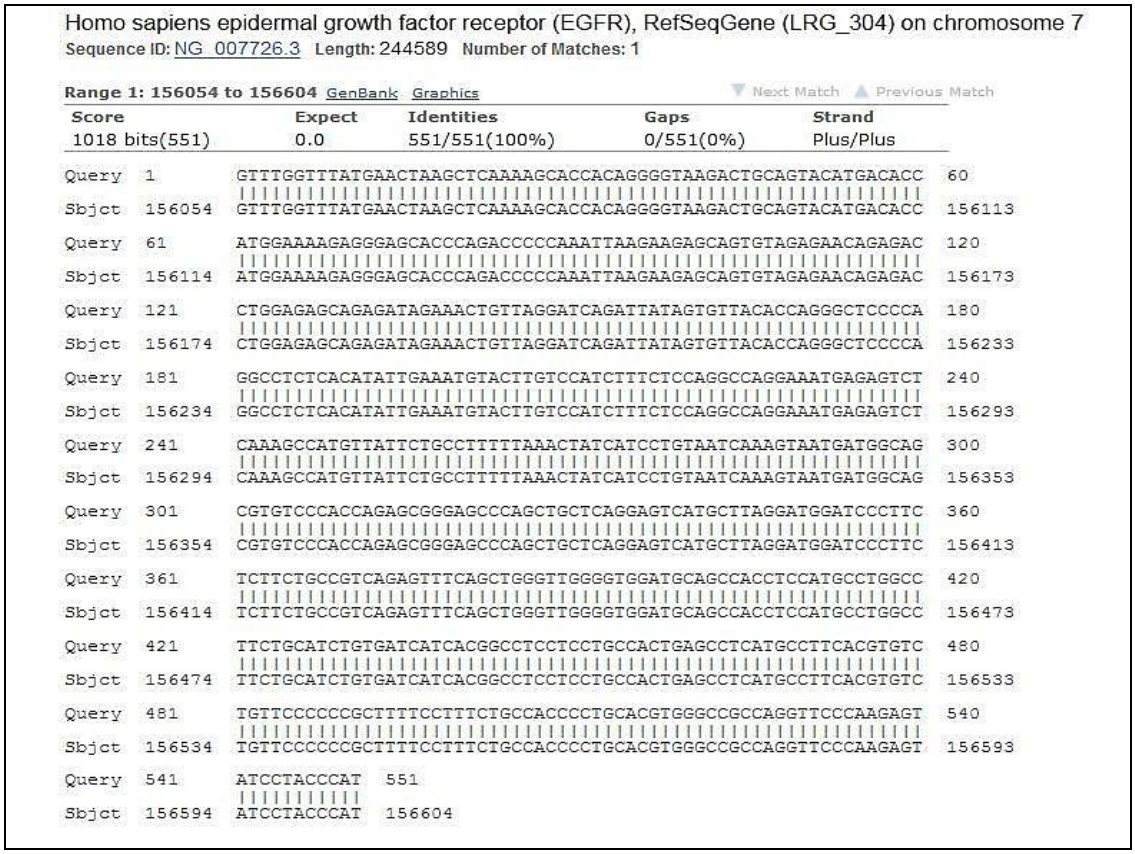

Figure 3.6: A BLAST analysis showing no mutation. All bases of the query match to the database.

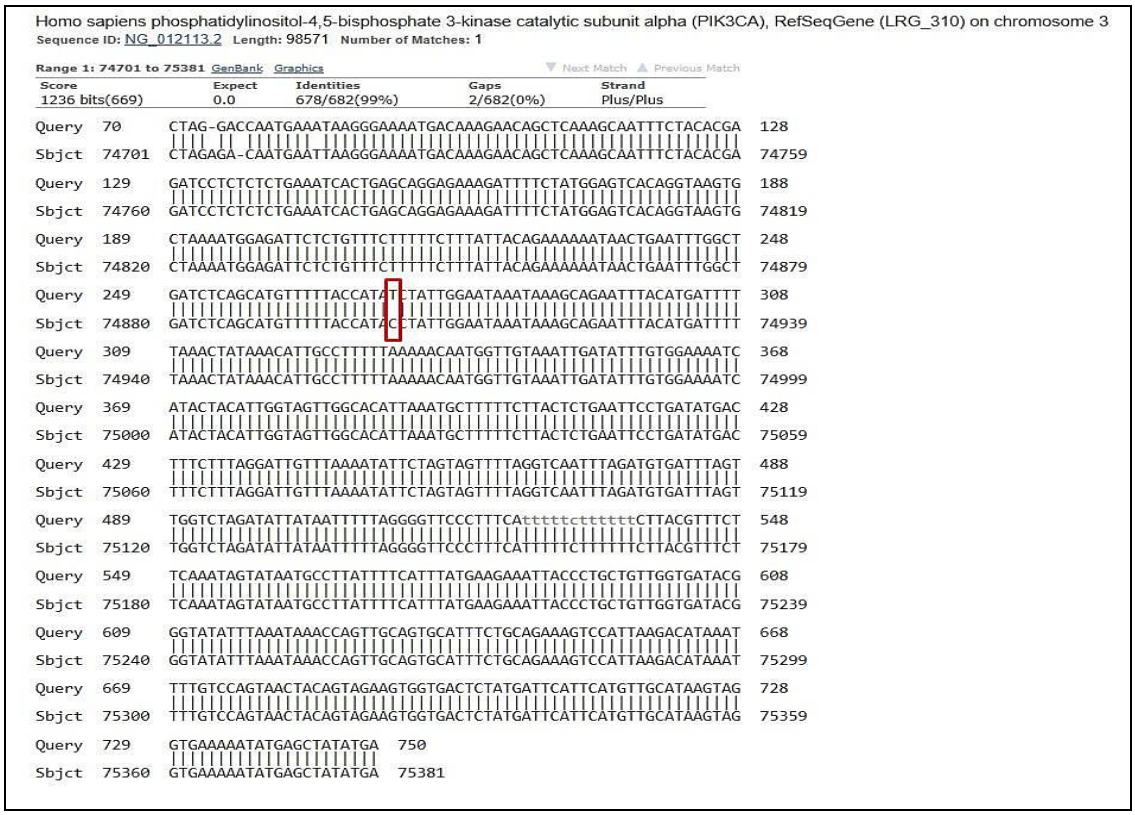

Figure 3.7: A BLAST analysis showing presence of a mutation. Red box indicates the mismatch.

3.2.3 Validation of mutation from chromatogram

The corresponding chromatogram was carefully inspected to ensure that the base change is not due to a sequence slippage but a valid mutation. If a clear peak was detected denoting one specific base, the sequence was acknowledged to be correct and base change was counted. Base changes for which clear single peaks did not exist were not counted as mutation. Mutation is confirmed from the chromatogram of second strand that was also sequenced.

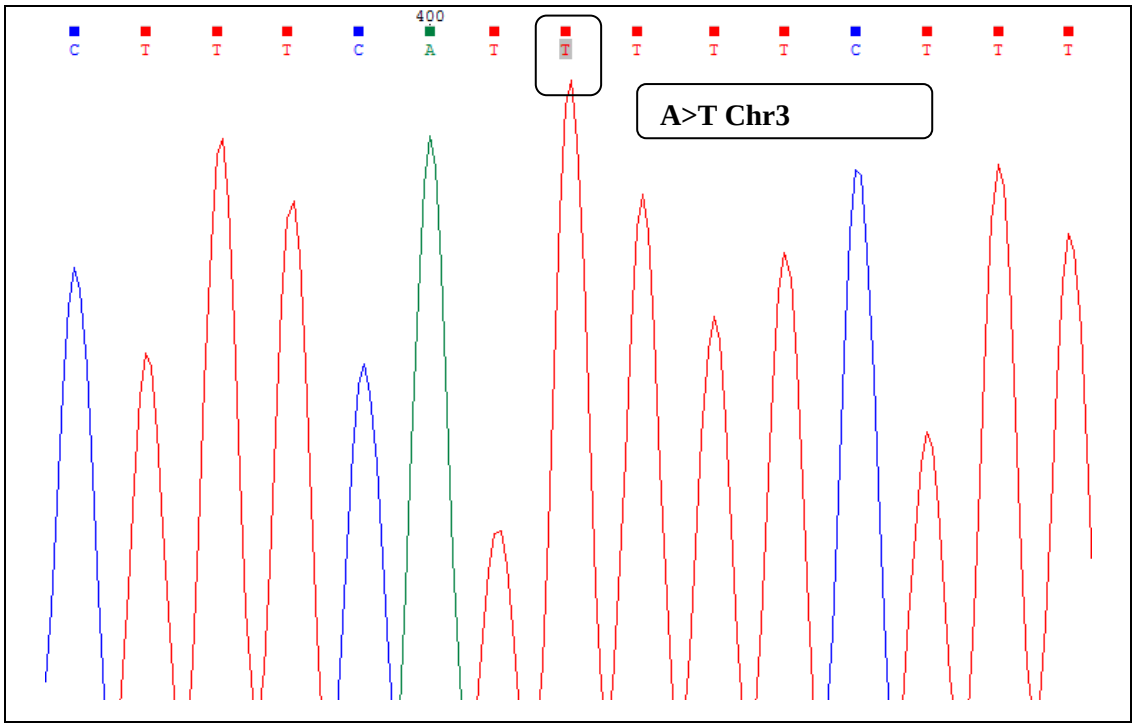

Figure 3.8: A chromatogram showing a base change (A>T)

As an example, a change in base (A>T) in position 179,218,425 of chromosome 3. The box marked peak is a single clear peak denoting T (Figure 3.8). Hence, this base change was counted as a valid mutation.

3.2.4 Result of sequence analysis

Using BLAST search, in total 39 mutations were found in 28 patients among the 46 patient samples (Table 3.1). The rest of the samples did not harbor any mutation along the region we sequenced.

Table 3.1: Detailed result of sequence analysis

Patient ID	Gene	Mutation	Mutation Type	Position (Chromosome) GRCh38.p7	Intron / Exon	Comment
T70	*PIK3CA*	T >A	Transversion	Chr3 179218237	Exon	523 Missense L>I
T108	*PIK3CA*	T >A	Transversion (htg)	Chr3 179234443	Exon	3'UTR
T70	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T104	*PIK3CA*	T > G	Transversion	Chr3 179218439	Intron	
T108	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T38	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T113	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T44	*PIK3CA*	G> A	Transition	Chr3 179218352	Intron	
T52	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T90	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T53	*PIK3CA*	T > G	Transversion	Chr3 179218439	Intron	
T100	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T40	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T47	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T51	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T71	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T101	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T90	*PIK3CA*	C >T	Transition	Chr3 179234223	Exon	Synonymous I>I
T55	*PIK3CA*	T>A	Transversion	Chr3 179234446	Exon	3'UTR
T90	*PIK3CA*	T>A	Transversion	Chr3 179234447	Exon	3'UTR
T108	*PIK3CA*	C >T	Transition	Chr3 179234232	Exon	1025 T>T Synonymous
T38	*PIK3CA*	C >T	Transition	Chr3 179234232	Exon	1025 T>T Synonymous
T70	*PIK3CA*	C >T	Transition	Chr3 179234232	Exon	1025 T>T Synonymous
T97	*PIK3CA*	C >T	Transition	Chr3 179234232	Exon	1025 T>T Synonymous
T45	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T58	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T60	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR

Patient ID	Gene	Mutation	Mutation Type	Position (Chromosome) GRCh38.p7	Intron / Exon	Comment
T97	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T106	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T109	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T115	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T51	*EGFR*	G> A	Transition	Chr7 55170193	Intron	
T51	*EGFR*	G> A	Transition	Chr7 55170210	Intron	
T47	*EGFR*	Insertion (A)	Addition	Chr7 55170230	Intron	
T47	*EGFR*	A>G	Transition	Chr7 55170332	Exon	636 Mis-sense M>V
T91	*KRAS*	G>A	Transition (htg)	Chr12 25245246	Intron	
T84	*KRAS*	A>C	Transversion (htg)	Chr12 252271121	Intron	
T108	*KRAS*	A>C	Transversion (htg)	Chr12 252271121	Intron	
T110	*KRAS*	A>C	Transversion (htg)	Chr12 252271121	Intron	

Note: Htg = heterozygous, chr = chromosome

In summary, our data show that among 46 samples, 18 did not harbor any mutation (39.13%), 22 samples harbored mutation in one gene fragment (47.83%) whereas 6 samples harbored mutations in more than one gene fragments (13.04%) (Figure 3.9).

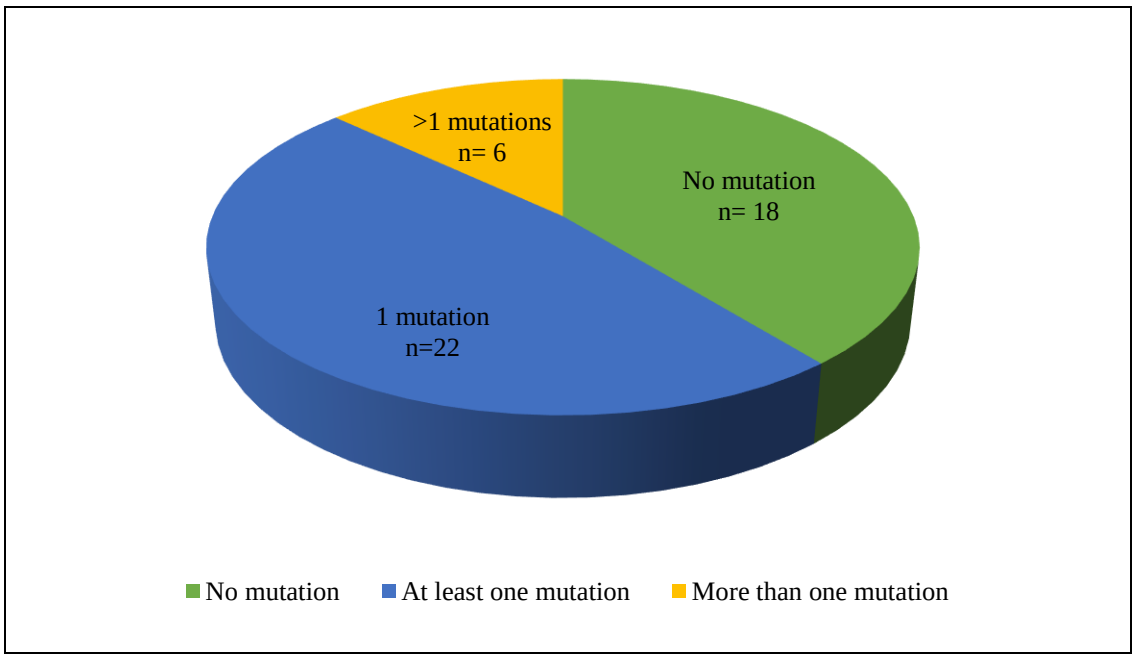

Figure 3.9: Distribution of cases by presence of mutation.

3.2.4.1 Individual gene mutation result

Amongst the 46 samples, it has been found that *PIK3CA* was the most frequently mutated gene (in 52.17% patients), *EGFR* gene was the second most mutated gene (in 23.91% patients) and finally *KRAS* gene was found to be the least mutated in the tested patients (in 8.7% patients) (Table 3.2).

Table 3.2: Individual gene mutation result

***KRAS* Gene**	***EGFR* Gene**	***PIK3CA* Gene**
4 in 46 samples	11 in 46 samples	24 in 46 samples
8.7%	**23.91%**	**52.17%**

3.3 Comparison with previous studies

Some studies have been done on individual gene mutations in cervical cancer in other parts of the world. Study of Wright *et al*. (Alexi A. Wright et al., 2013a), have been compared with this study (Figure 3.10). A much higher percentage of *PIK3CA* mutation was observed in this study compared to the previous one (52.17% in this study vs 31.3% in the previous study). Similarly, *EGFR* mutation rate was found to be much prevalent in Bangladeshi patients compared to the statistics of Wright *et al.* (23.91% in this study vs. 3.8% in the previous study). However, not much of a difference has been observed for the *KRAS* gene mutation (8.7% in this study vs.8.8% in the previous study) (Figure 3.10).

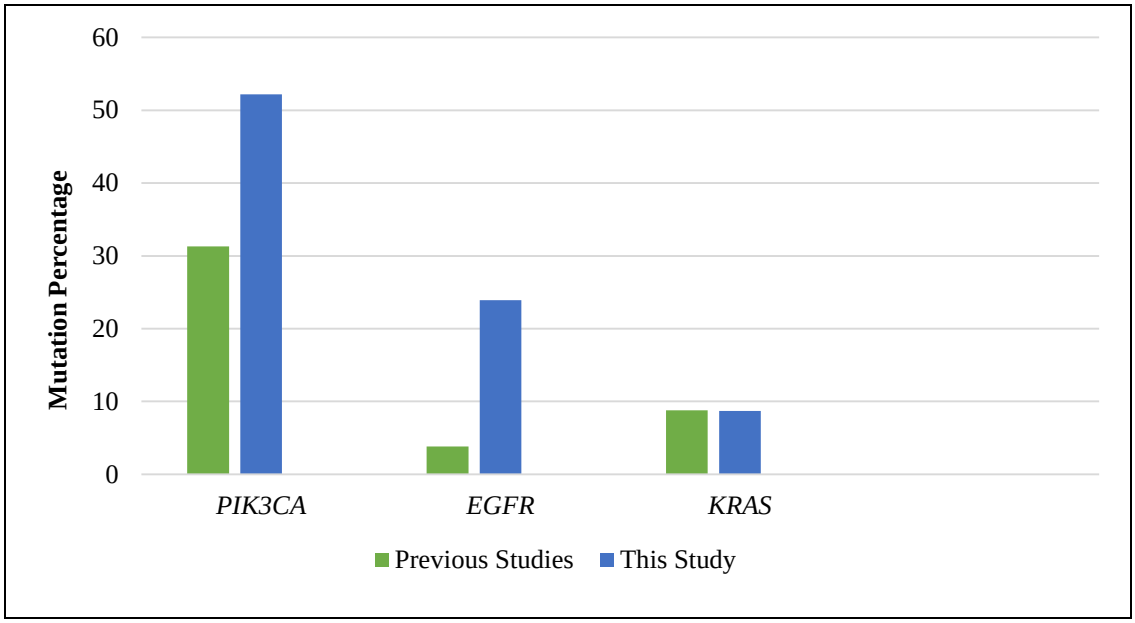

Figure 3.10: Comparison between previous works (Wright *et al.*, 2013) and this study.

3.4 Mutation analysis

3.4.1 Analysis of effect of exonic and intronic mutation on gene function

Among the 39 mutations, 17 were unique. These unique mutation were analyzed using Variant Effect Predictor tool. All 17 different mutations in the 3 target genes showed significant low LoFtool scores (Table 3.3) which can be used to predict that all these mutations can have probable damaging effects and make the patients more vulnerable to carcinogenesis and metastatic development.

Table 3.3: Prediction of the putative effect of the mutation on gene function.

Gene	Mutation	Intron / Exon	Position (Chromosome)	LoFtool Score	Mutation Effect
EGFR	G> A	Exon	Chr7 55170575	0.0455	Probably Damaging
	G> A	Intron	Chr7 55170193	0.0455	Probably Damaging
	G> A	Intron	Chr7 55170210	0.0455	Probably Damaging
	Insertion (A)	Intron	Chr7 55170230	0.0455	Probably Damaging
	A>G	Exon	Chr7 55170332	0.0455	Probably Damaging

Gene	Mutation	Intron / Exon	Position (Chromosome)	LoFtool Score	Mutation Effect
KRAS	G>A	Intron	Chr12 25245246	0.19	Probably Damaging
	A>C	Intron	Chr12 252271121	0.19	Probably Damaging
PIK3CA	T >A	Exon	Chr3 179218237	0.268	Probably Damaging
	T >A	Exon	Chr3 179234443	0.268	Probably Damaging
	C >T	Intron	Chr3 179218425	0.268	Probably Damaging
	T > G	Intron	Chr3 179218439	0.268	Probably Damaging
	G> A	Intron	Chr3 179218352	0.268	Probably Damaging
	T>A	Intron	Chr3 179234011	0.268	Probably Damaging
	C >T	Exon	Chr3 179234223	0.268	Probably Damaging
	T>A	Exon	Chr3 179234446	0.268	Probably Damaging
	T>A	Exon	Chr3 179234447	0.268	Probably Damaging
	C >T	Exon	Chr3 179234232	0.268	Probably Damaging

3.4.2 Exonic mutation frequency

Seventeen mutations were found to be exonic among the 39 mutations and rest others were intronic. Among the exonic mutations, two mutations were found to be non-synonymous, one was found in the *PIK3CA* gene and other one in the *EGFR* gene. None of the mutations in the *KRAS* gene were exonic. *PIK3CA* gene was found to contain a high amount of exonic mutations (72.7%) than the *EGFR* gene (37.5 %) (Table 3.4).

Table 3.4: Frequency of mutation found in exon

Gene	Total mutation	Exonic mutation	Number of non-synonymous mutation	Intronic mutation	% of exonic mutation
PIK3CA	24	9	1 out of 9	15	37.5%
EGFR	11	8	1 out of 8	3	72.7%

3.4.3 Determination of the location of mutant amino acid in the protein

Using MutationMapper tool (Vohra & Biggin, 2013) the particular domain where the target mutant amino acid is located were found. In EGFR protein, mutant amino acid (valine) is present in a functional domain named Growth Factor Receptor 4 domain (Figure 3.11). Missense mutation of PIK3CA protein was located in the beginning of the PIK domain (Figure 3.12), which is conserved in all PI3 and PI4-kinases and role of this domain unclear but it has been suggested to be involved in substrate presentation (Flanagan et al., 1993).

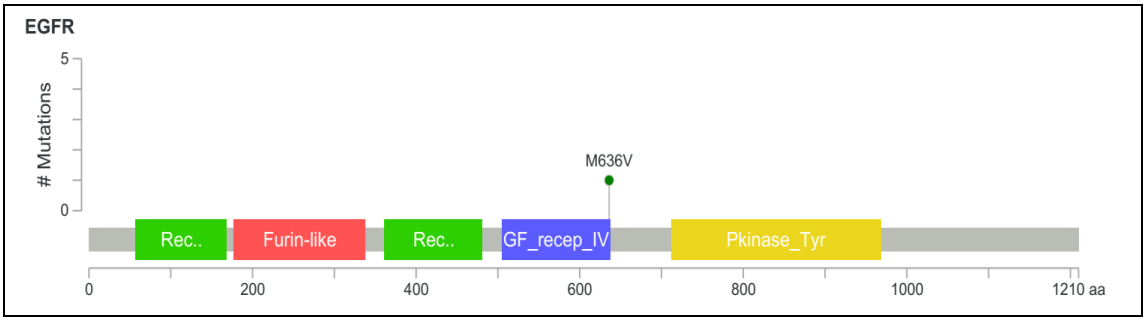

Figure 3.11: Location of the mutant amino acid in the EGFR protein structure.

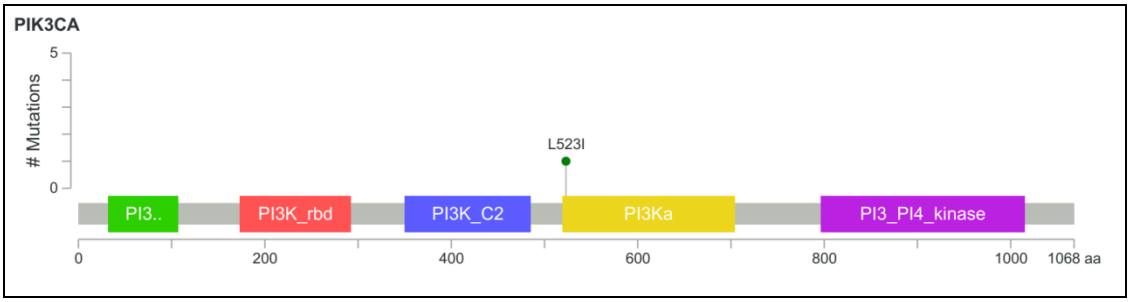

Figure 3.12: Location of the mutant amino acid in the PIK3CA protein structure.

3.4.4 Analysis of the effect of non-synonymous mutation on protein

SIFT scores for the non-synonymous mutations are found to be high and PolyPhen scores are found to be lower for both genes (Table 3.5), predicting the structure and function of the proteins can retain its natural states even though the mutation occur to the change the particular amino acid.

Table 3.5: Prediction of the effect of the non-synonymous mutation on protein structure and function.

Gene	Mutation	Intron / Exon	Position (Chromosome)	SIFT Score	PolyPhen Score
EGFR	G> A	Exon	Chr7 55170575	0.5 (tolerated)	0 (benign)
PIK3CA	T >A	Exon	Chr3 179218237	0.26 (tolerated low confidence)	0.001 (benign)

Due to the low delta G value for the both of the non-synonymous mutations, MuPro tool predicted that both of the protein structures may lose its stability upon the accumulation of the particular variant amino acid instead of the wild-type one (Table 3.6).

Table 3.6: Mutant protein's stability prediction.

Gene	Mutation	Intron / Exon	Position (Chromosome)	MuPro Score (Delta delta G value)	MuPro Prediction
EGFR	G> A	Exon	Chr7 55170575	-1.2135263	Decrease stability of protein structure
PIK3CA	T >A	Exon	Chr3 179218237	-1.0116756	Decrease stability of protein structure

3.4.5 Detection of change of amino acid and its effect in protein 3D structure

All the nucleotide sequences were translated into amino acids to find out whether there any crucial amino acid was changed due to mutation, and also if such amino acid change may lead to significant change in 3D protein structure. Structure of mutated proteins was determined by homology modeling using the tool SWISS-MODEL.

Among the 2 non-synonymous mutations, first one was in *EGFR* gene (chromosome 3) at position 55,170,332. The A to G base transition has resulted in an amino acid change from methionine to valine at amino acid position 636. This amino acid change was used to predict change in protein structure using SWISS-MODEL and as a result, no

significant structural difference has been observed due to that particular amino acid substitution (Figure 3.13).

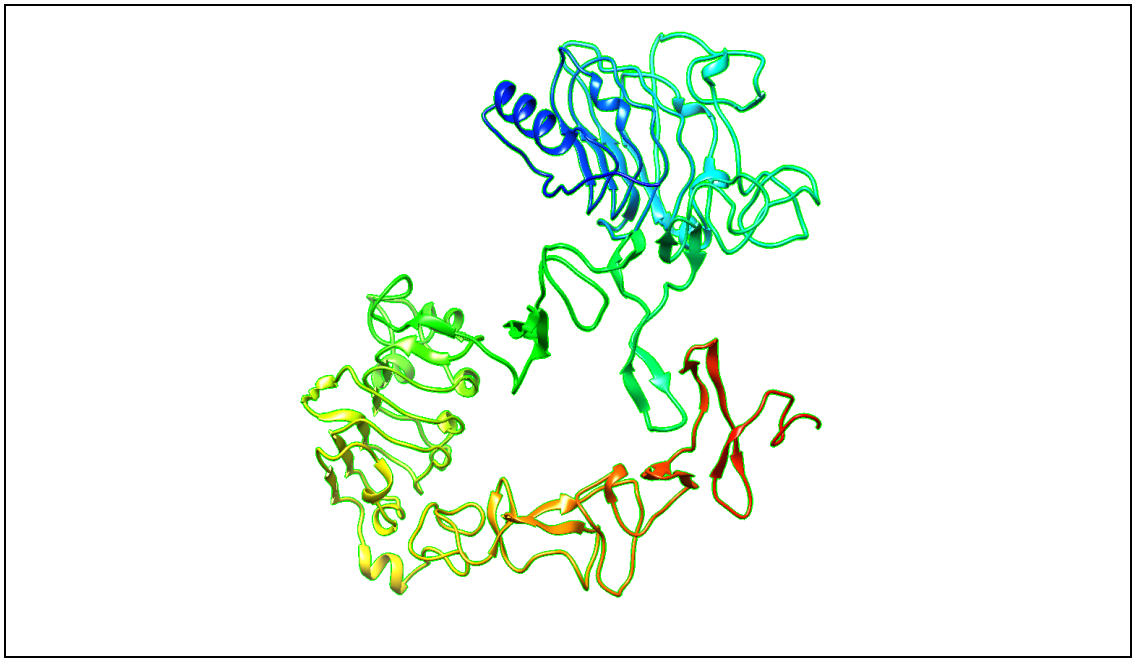

Figure 3.13: EGFR wild-type protein structure.

The second mutation was in *PIK3CA* gene (chromosome 3) at position 179,218,237. Here, the T to A base transition has resulted in an amino acid change from leucine to isoleucine at amino acid position 523. This amino acid change caused a slight change in PI3 and PI4-kinases domain in 3D structure (Figure 3.14).

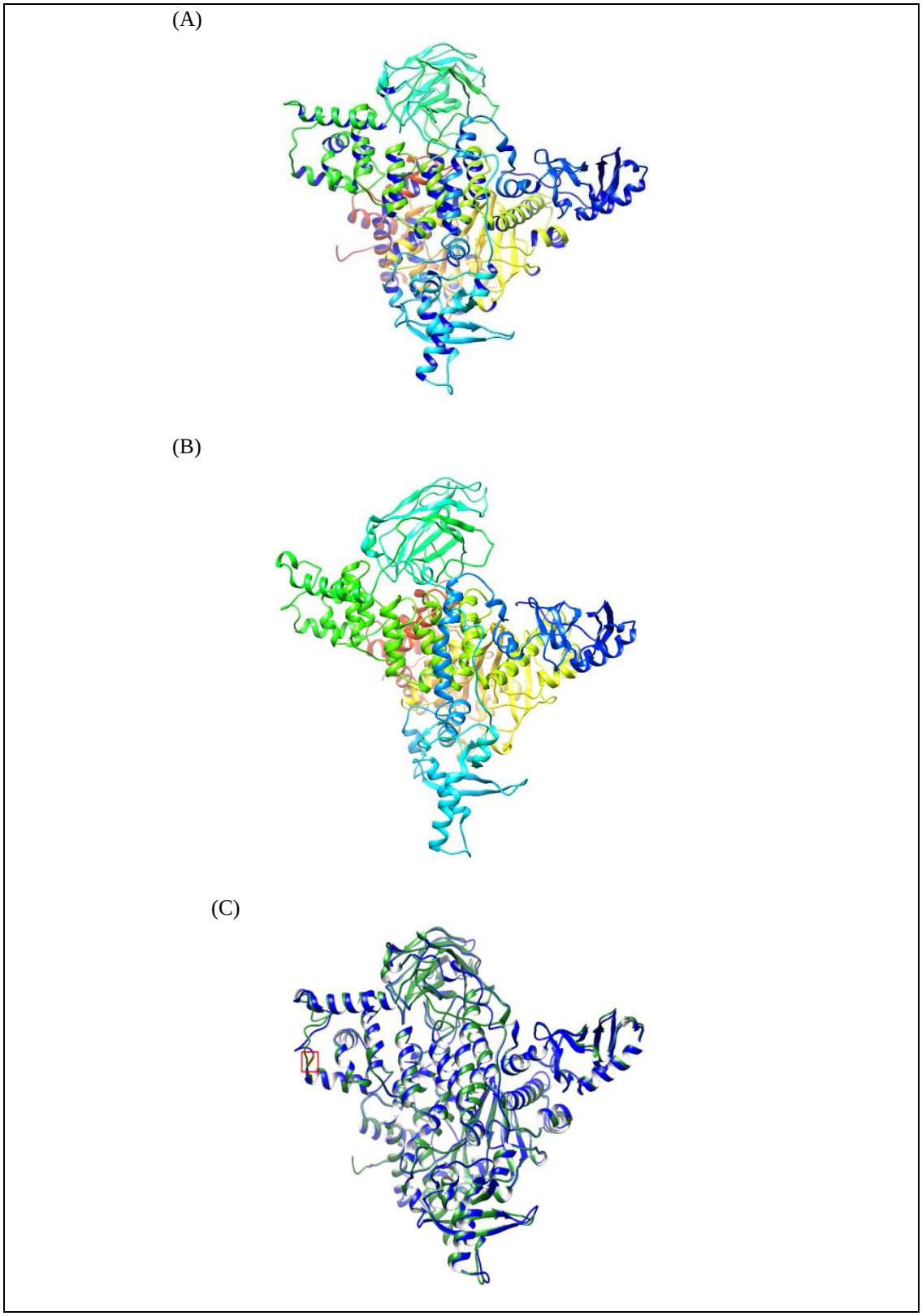

Figure 3.14: 523L>I mutation in *PIK3CA*. (A) The wild-type protein, (B) the mutated protein and (C) the wild-type and the mutated protein are overlapped where the marked red box shows the mutation position in PIK3CA protein structure.

CHAPTER FOUR

DISCUSSION AND CONCLUDING REMARKS

4.1 Discussion

Cervical cancer is one of the most frequent cancer in women in 2012 representing 7.9% of all female cancers (WHO). Approximately 90% of the 270,000 deaths from cervical cancer in 2015 occurred in low and middle-income countries (WHO). This is the most common cause of female death by cancer in 43 countries. The estimated global economic burden of cervical cancer in 2009 was 3 billion US dollars.

Cancer causing infections, such as hepatitis and human papilloma virus (HPV), are responsible for up to 25% of cancer cases in low- and middle-income countries. Vaccination against these HPV and hepatitis B viruses could prevent 1 million cancer cases each year (Plummer, de Martel et al. 2016). HPV is recognized as the most important risk factor for cervical cancer for last 15 years (zurHausen and De Villiers 1994). World health organization recognized sexually transmitted HPV infection as a modifiable and avoidable risk factor.

Bangladesh has a population of 54.38 million women ages fifteen years and older who are at risk of developing cervical cancer. Current estimates indicate that every year 11,956 women are diagnosed with cervical cancer and 6,582 die from the disease (HPV Information Center). Cervical cancer ranks as the second most frequent cancer among women between 15 and 44 years of age in Bangladesh (WHO). In Southern Asia, the region Bangladesh belongs to, about 7.9% of women in the general population are estimated to harbor cervical HPV infection at a given time and 82.8% of invasive cervical cancers are attributed to HPV 16 or 18 (Bangladesh-ICO Information Centre on HPV and Cancer, 2014). Vaccination program in Bangladesh is in its primary steps, and vaccine is costly, with approximately 1,600 taka per dose.

Mutation that can induce neoplastic transformation is known as oncogenic mutation and can produce an abnormal protein or change expression level of the protein coded by the gene harboring the mutation. This abnormal or overexpressed protein can be a potential target of drugs. These drugs can eventually destroy cancer cell or modify the disease process. In this perspective, researchers are trying to find out mutation specific for certain cancer in last few decades. A classic example is the translocation of gene segment between two chromosomes resulting in BCR-ABL fusion protein found in chronic myeloid leukemia (CML). A Tyrosine kinase inhibitor named imatinib which

inhibits this protein is targeted therapy for CML. This study attempted to identify mutations in genes which can be specific for cervical cancer.

In our study cervical tissue samples have been collected from patients of Department of Gynecological Oncology, National Institute of Cancer Research and Hospital situated in Mohakhali, Dhaka. Patients were provided with a consent form in order to get permission to use their tissue sample. A total of forty-six tissue samples were used to perform this study. Histopathology of the samples revealed 2 adenocarcinoma cases and all other cases are squamous cell carcinomas.

In this study, we amplified specific gene fragments. *EGFR*, *KRAS* and *PIK3CA* genes were selected to be amplified in specific regions since these genes are implicated in most cancers, especially in cervical cancers. Total 5 pairs of primers were used. Two different sets of primers were chosen to amplify two different sites on *PIK3CA* and *KRAS* genes since these two genes were to harbor more than one mutation hotspot. DNA extraction was carried out and gene fragment sequencing was done for all amplified products. After sequencing, each sequence was matched with the existing sequence data in the NCBI (National Center for Biotechnology Information) database. Any disparity from the database's sequence was noted down and checked with the corresponding sequence's chromatogram. If a clear single peak was found, the base change was noted as a mutation. If no clear peak was found or more than one peak existed at the same place, it was not counted as a mutation. This is because an undistinguished peak usually denotes an error in sequencing. All the amplified products were sequenced using the forward and the reverse primers. Both sequences were matched to ensure correct mutation detection.

We observed that, among 46 samples, 28 samples harbored mutation (60.86%). The other samples did not harbor any mutation. Among the 28 samples which harbored mutation, 22 samples harbored mutation in one gene (78.57%) whereas 6 samples harbored mutations in more than one genes (21.43%). In total, 39 mutations were found.

Eleven different mutations were found in amplified *EGFR* gene fragments, among which 1 specific mutation was found in more than one patient samples (in 7 samples). On the other hand, 24 different mutations were found in *PIK3CA* gene fragment amplicons, among which 2 mutations were found in 6 patients. Four different mutations were found in *KRAS* gene fragment amplified products.

Almost all of the mutations identified were base substitution. Both transitions and transversions were found. Only one insertion mutation was found in *EGFR* gene fragment at chromosomal position 55,170,230 where an adenine was added.

Our study shows that Bangladeshi patients have *KRAS* mutation frequency (8.7%) similar to that reported by Wright *et al.* (Alexi A. Wright et al., 2013a) (8.8%). A 7% mutation in *KRAS* was also reported by Spaans *et al*., 2015. Iida *et al*., (Iida et al., 2011) also reported somatic mutations in *KRAS* in 3 (6.3%) of 48 cervical adeno/adenosquamous cell carcinomas.

However, surprisingly, *EGFR* mutation frequency is over 6 times higher in our patients (23.91% versus 3.8%) and *PIK3CA* mutation frequency is over 1.6 times (52.17% versus 31.3%) compared to study of Wright *et al*., 2013 and 2.6 times higher (52.7% versus 20%) compared to Spaans *et al*., 2015(Spaans et al., 2015), report. Even *PIK3CA* mutation rate is 3.8 times higher than those in Chinese patients (13.6%) as reported by Xiang *et al*., 2015(Xiang et al., 2015).

Survival of patients with *KRAS* mutation is poorer than in patients without *KRAS* mutations (Jiang et al., 2017), therefore, a combination of *KRAS* mutation detection and HPV genotyping would be useful in identifying a patient with poor prognosis for further interventions. Among the three most common histological subtypes of cervical cancer (squamous cell carcinoma (SCC), adenocarcinoma (AC), and adenosquamous carcinoma (ASC)) *KRAS* mutations are reported to occur more frequently in AC than SCC (Spaans et al., 2015). The same study also observed worse disease-free survival (HR 1.57, *P*=0.043) in positive *KRAS* mutation cases. A set of endometrial-like cervical cancers comprised predominantly of HPV-negative tumors and characterized by mutations in *KRAS*, *ARID1A* and *PTEN* was discovered in a study conducted by (Network, 2017).

EGFR is a membrane tyrosine kinase receptor that is known to contribute to the growth activity and tumor survival, and hence this has become a therapeutic target in several cancers. The extracellular EGFR-binding domain is usually targeted with monoclonal antibodies such as cetuximab or panitumumab, and inhibition of the EGFR tyrosine kinase activity is done with other small molecules as well such as gefitinib and erlotinib, especially patients who have specific functional *EGFR* mutations. In *EGFR* gene, exons 18–21 are the hot spot region for gain-of-function mutations. Previous studies (ARIAS-

PULIDO et al., 2008; Iida et al., 2011) found a strong correlation between poor prognosis and *EGFR* gene amplification in patients with cervical squamous cell carcinoma. In other carcinomas like leukemia, glioblastoma, and colorectal, gastric, breast, and hepatocellular carcinomas *EGFR* mutation frequency reported to be low. Neither Iida *et al*., 2011; nor the study of Arias-Pulido *et al*., 2008 found presence of *EGFR* mutations in exons 19 and 21. No mutations identified in their samples affecting the EGFR kinase domain in exons 18 through 21 in human neoplastic samples analyzed. However, in cervical carcinoma, we have found a mutation in our Bangladeshi patients. These suggests that mutations in the EGFR kinase domain may be not common in other part of the world. Our results suggest, therefore, that treatment of CC patients with TKIs needs mutational screening before prescribing drugs and may not have the same efficacy as seen in patients with no-mutation. Therefore CC patients without such mutation, targeting the *EGFR* with other inhibitors may be more appropriate.

PIK3CA mutations can cause the deregulation of the phosphatidylinositol 3-kinase-Akt signaling pathway, which comprises cell proliferation, transformation, and cell survival, stimulating oncogenesis. Aberrations in this pathway are described in various cancers, including cervical cancer, and this has led to the development of PI3K-inhibitors and Akt-inhibitors as potential cancer therapies, with some already having reached clinical trials. *PIK3CA* mutation rates are very heterogeneous in different studies (20–37%) (de la Rochefordiere et al., 2015; McIntyre et al., 2013). However, Bangladeshi patients harbor more mutations (52.17%). In Spaans *et al*., 2015 (Spaans et al., 2015) study, a clear trend was seen for reduced survival in patients carrying a *PIK3CA* mutation, especially with the SCC subtype. Similarly Wright *et al.*, 2013 (Alexi A. Wright et al., 2013a) also showed that an association lies between *PIK3CA* mutation and shorter survival.

After DNA sequence analysis, corresponding amino acid analysis was done to find out the effect of mutations on proteins. Among the 39 different mutations, 17 mutations were located in exon regions. Nine of them were *PIK3CA* mutations and the other 8 were *EGFR* gene mutations. The other 21 mutations were in intron position, hence, they did not have any effect on the amino acid sequence.

The 17 mutations of exon regions were analyzed and it was found out that 15 of them were synonymous. This means that the change in nucleotide base did not change the amino acids produced by their codons. One mutation (A>G) in *EGFR* gene

(chromosome 7) at chromosome position 55,170,332 produced a non-synonymous change, that resulted in a methionine to valine substitution at amino acid position 636 of the protein.

We have done different bioinformatics analysis of mutations we have found using tools like Variant Effect Predictor tool, MuPRo tool, Mutation Mapper tool. LoFtool scores of all intronic and exonic mutations which were significantly low, predicts that effect of the mutation on the functionality of the gene is probably damaging. SIFT and polyphen scores showed that effect of the non-synonymous mutation on the function of protein is not that much significant. But the stability of protein may be affected which is predicted by MuPRo tool.

The mutant variant of *EGFR* is found in the isoform 3 (705 amino acids) which differs from the canonical sequence as it has different amino acid composition from position 628 to 705 and does not have the amino acids from 706 to 1210. So, the predicted position of the mutation in the canonical sequence which located at the GF-receptor domain IV will not be same for the isoform 3. To find out the structural changes, mutant protein was modeled and aligned with the wild-type isoform-3 but no structural difference was observed; this mutation may make the protein unstable rather than altering its structure of the functional domain. So we observed a structural change of the protein caused by non-synonymous mutation only in *PIK3CA* gene.

3D structure changing due to the mutation (T>A) observed in *PIK3CA* gene on chr3 position 179,218,237. The T to A base transition has resulted in an amino acid change from leucine to isoleucine at amino acid position 523. This change alters the protein structures in PI3 and PI4-kinases domain. *In vitro* protein level analysis needs to be done to find out the exact effect of this amino acid change.

4.1.1 Focal points of analysis

Several points are of special importance concerning this study. These are listed below:

- Almost all the mutations were base substitution mutations.
- Both transition and transversion mutations existed.
- Some mutations were found in more than one patient samples.

- *PIK3CA* mutation was the highest, followed by *EGFR* and *KRAS* mutation.
- Seventeen mutations were found to be in exon region.
- Two non-synonymous (missense) mutation were found, one in *EGFR* gene where methionine was changed to valine and another non-synonymous mutation was found in *PIK3CA* gene, where leucine was changed to isoleucine. The affected domain are PI3 and PI4-kinases.

4.2 Concluding remarks

Cervical cancer is a major cause of morbidity and mortality, particularly in developing countries. This study reveals that somatic mutations exist in cancer tissues of cervical cancer patients. Affected women are usually, working, and raising children, which creates substantial social problems. The data obtained from this study can be used to establish a mutation database for Bangladeshi cervical cancer incidents. Since the findings suggest that cervical cancer may harbor targetable oncogenic mutations, this should encourage further studies to better understand these mutations and exploit them for clinical use.

Nowadays, many cancer diagnoses apply specific mutation detection. Specific mutations for specific cancers are also being exploited for more tailored treatment strategies. Future studies are needed to validate this finding and to explore the biological and clinical importance of these mutations. Precise classification of cervical carcinomas in combination with mutation profiling is valuable for predicting disease outcome and may guide the development and selection of tumor-specific treatment approaches.

4.2.1 Future endeavors

This study was conducted as basic research and as such, is not above limitations. More studies need to be carried out on this topic in order to establish a statistically significant database. Important prospects to further the studies include the following.

- More samples need to be analyzed for conclusive data.

- Also, the exact impact of the identified mutations needs to be found out. For this purpose, protein level studies need to be carried out.
- Lastly, whether these mutations can be used for diagnostic purposes or for more tailored treatment strategies need to be found out.

Despite limitations, there are reasons to believe that this study, both in terms of its conclusions and robust methodology, can serve as the first step to more comprehensive analyses of oncogenic mutations in cervical cancer patients in Bangladesh.

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APPENDIX I

Buffers and Reagents

A. Proteinase-K (10 mg/mL)

50 mg of proteinase-K was dissolved in 5 mL TE-buffer.

B. 70% ethanol

70 mL ethanol was added to 30mL distilled water and stored at room temperature.

C. 10x-TBE (Tris-borate –EDTA, pH 8.0)

108 gm of Tris-base, 55 gm of boric acid and 40m1 of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 1000 mL. The buffer was stored at room temperature.

D. 0.5 M EDTA

18.61 gm ofNa2EDTA.2H2O (disodium ethylene diamine tetra-acetic acid) was dissolved in 80 mL of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. The final volume was made up to 100 mL with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

E. 1x-TE buffer (Tris-EDTA)

TE buffer (10 mMTris-Cl/1mM EDTA, pH 8.0) was prepared was prepared by diluting concentrated stocks of 1 M Tris-Cl and 0.5 M EDTA in distilled water. For making 1000mL or 1L of TE buffer, 10 mL of 1 M Tris-HCl (pH 8.0) and 2 mL EDTA (0.5 M) and distilled water added up to 1000mL. The buffer was autoclaved and was stored at room temperature.

F. 6X-Gel loading buffer (Glycerol and bromophenol blue)

3mL glycerol (30%) was added to 25mg bromophenol blue (0.25%) and distilled waterwas added upto 10mL.

G. EtBr (0.5 µg/mL)

10 µL of 10 mg/mL Ethidium bromide solution was added to 200 mL distilled water. This solution was stored at room temperature and covered with aluminum foil.

H. Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na2HPO4 and 2.0 gm of KH2PO4 in 800 mL distilled water. pH was adjusted to 7.4 with HCL. The final volume was adjusted to 1 L by distilled water.

I. Buffer AW1 (Wash Buffer 1)

19 mL of Buffer AW1 concentrate (provided with the QIAamp® DNA Mini Kit Catalog No.51304) was mixed with 44 mL ethanol (96-100%) before using.

J. Buffer AW2 (Wash Buffer 2)

13 mL of Buffer AW2 concentrate (provided with the QIAamp® DNA Mini Kit Catalog No.51304) was mixed with 43 mL ethanol (96-100%) before using.

K. BufferB3 (Binding Buffer solution)

For every 21.7 mL B3 (supplied with Invitrogen PCR DNA Purification Kit), 2.3 mL isopropanol (>99%) was added before using.

L. BufferW1 (Wash buffer)

For every 16 mL Wash Buffer (supplied with Invitrogen PCR DNA Purification Kit, 64 mL ethanol (96-100%) was added before using.

APPENDIX II

Instruments

The important equipment used throughout the study are listed below:

Autoclave, Model no: HL-42AE	Hirayama corp, Japan
Automated thermocycler, Model: 12137	Bio-Rad, USA
Centrifuge, Mode1:5804	Eppendorf, Germany
Class II Microbiological safety cabinet	Labcaire, USA
Electric balance, Scout, SC4010	USA
Freezer (-30°C)	Liebherr, Germany
Gel documentation	Sigma, USA
Horizontal gel electrophoresis apparatus Hl-SET	UK
Incubator	Japan
Microcentrifuge, Mikro20	Germany
Micropipettes	Eppendorf, Germany
Microwave oven, Model: D90N30 ATP	Butterfly, China
pH meter, Model no: MP220	Eppendorf, Germany
Power pack	Toledo, Germany
Refrigerator (4°C)	Vest frost
Room temperature horizontal shaker	Gerhardt, Germany
Sterilizer, Model no: NDS-600D	Japan
Water bath, Model: SUM	England

APPENDIX III

CONSENT FORMS

Consent form for Specimen (tissue and blood) and Data collection for research purpose

Study title: Detection of mutation and polymorphism in marker genes of cervical carcinoma in Bangladeshi patients.

INVESTIGATORS:

1. Dr. Abul Bashar Mir Md. Khademul Islam, Principal Investigator,
   Associate Professor, Department of Genetic Engineering and Biotechnology,
   University of Dhaka, Dhaka-1000, Bangladesh.

2. Dr. Mahmuda Yasmin, Co-Investigator,
   Professor, Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh.

3. Professor Dr. Sabera Khatun, Co-Investigator,
   The Head, Gynecological Oncology Unit, Department of Obstetrics and Gynecology,
   Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka.

PURPOSE:

As cervical cancer is prevalent in Bangladesh, mortality rate is high in the context of low socioeconomic condition, we are experiencing an increasing burden of this disease; so there is indication to conduct genetic research in our country. We will perform molecular profiling including mutation detection, gene expression, HPV genotype testing and blood protein marker detection**.** Outcome of our result can help clinicians in prevention and early diagnosis, understanding of prognosis & intervention in treatment modalities of this cancer.

RISKS AND DISCOMFORTS:

Donating blood or specimens for research may have little risk. Researchers believe the chance of this risk is very small and protections are in place to lessen this risk. Even then, during biopsy, patients may feel some pain. Some unwanted complications may arise while collecting the tissue samples. All personal medical information about donor and any information obtained from this study of donor's specimen will be preserved in a secured database. Donor's name and identity will be used only for data collection and will not be disclosed to a third party. All reasonable efforts will be made to protect the confidentiality of information that can in any way be connected to donor.

BENEFITS:

Taking part in this research will not benefit to donor directly; however, what we learn may help others in the future.

ALTERNATIVES:

Donor may choose not to take part in this study.

COSTS:

Donor will bear his/her own treatment expenditure. There will be no cost to donor for any procedures required for the research. Donor will not be paid or given any other award for taking part.

PROPERTY DONATION:

By agreeing to take part, you allow the use of your samples for the research described in the purpose section of this document. In addition, you agree that we may make any lawful use of your samples, including future research studies, destroying them, or transferring them to a public or private entity.

CONFIDENTIALITY:

Every effort will be made to keep donor's information records private. All others, including employers, insurance companies, personal physicians, and relatives will be refused access to the information and to the samples, unless you provide written permission, or unless we are required by law to do so. Anything that can identify you will be kept in private, protected files. An ID number will be assigned to you, your tissue samples, and information about your medical history. Only the investigator named on this consent form will be authorized to link the ID number to your name. The link of your ID number to your name or any other identifying data will be stored in the established secure database. Any future research done on any of the samples must be designed in a way that protects your privacy and presents research results and data anonymously. The Institutional Review Board must also monitor it.

SPONSORSHIP:

The possible funding agencies for this study are Ministry of Science and Technology, Govt. of Bangladesh and University Grant Commission of Bangladesh.

CONFLICT OF INTEREST:

The principle investigator or the co-investigators and the other possible research team members have no conflict of interest to declare.

PARTICIPATION:

Fatima Tuj Zohura, Cell Phone: xxxxxxxxxx has offered to answer any other questions donors may have about this study. If donor has any questions regarding his/her rights as a research subject, he/she may contact us. If in the future donor decide no longer to take part in this study, we will destroy all identifying information and will not use your tissue in any future research. However, donor's tissue samples are already being used in current research project and if their withdrawal jeopardizes the success of the entire project, we may continue to use them until the project is completed. Donor will be given

a copy of this consent form for his/her records. Donor's signature below indicates that donor has red this consent form and agree to take part in this study.

CONSENT:

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of taking part in this study. I understand that I have the right to refuse to take part in the study. My decision about taking part in the study will not affect my health care.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my medical records will be kept private except as described to me.
6) I understand that no information about me will be given to anyone or be published without first asking my permission.
7) I hereby knowingly and voluntarily authorize you to use my specimen and my Protected Health Information in the manner described in this Consent Form.
8) I agree, or consent, that I may take part in this study.

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Date Subject's Signature

Witness-1:

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Date Signature

Witness-2:

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Date Signature

APPENDIX IV

DATA COLLECTION SHEET **Date:**

Detection of mutation and polymorphism in marker genes of cervical carcinoma in Bangladeshi patients.

ID		Urban	Rural	Name		Cell	

Age		Marital status	M	UM	Education				
					Illiterate	Literate	Secondary	H. secondary	Above

Address		Monthly income	Tk	Sanitation	

Menorrhagia		Postcoital Bleeding		Postmenopausal Bleeding		Intermenstrual Bleeding		Continued Bleeding	
Y	N	Y	N	Y	N	Y	N	Y	N

Pain		Weight Loss		Menarche	
Y	N	Y	N		

M	F	Age at time of marriage		AL C		P		G		1st PG	Yam	2nd PG	Yam	3rd PG	Yam

Smoking		OCP		Injection HC		Betel nut		F/H		Hb	ESR
Y	N	Y	N	Y	N	Y	N	Y	N	gm/dl	Mm

TC	cmm	N	%	L	%	LFT		Creat	mg/dl	RBS	mg/dL

Histo pathology	SCC	Adeno carcinoma	Other:	Informed about HPV vaccine	HPV vaccinated	Y	N

Staging		Informed about cervical carcinoma	Y	N

Treatment	Surgery	chemotherapy	Radiotherapy	Recurrence	Y	N

Comment	Signature of Investigator: